Novel Insights into Telomere Biology and Virulence Gene Expression in *Plasmodium falciparum*

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Summary

Plasmodium falciparum malaria is still one of the most preeminent and deadliest infectious diseases worldwide, imposing a tremendous health and economic burden on endemic countries. The high virulence of *P. falciparum* is mostly attributable to the expression of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) on the surface of infected red blood cells. PfEMP1 mediates intravascular parasite sequestration in vital organs, which contributes substantially to severe disease and death. Mutually exclusive transcription of the 60 *var* genes (encoding PfEMP1) and switching to formerly silenced variants results in antigenic variation and allows the parasite to efficiently evade host immune responses and to establish chronic infection.

Members of the *var* multigene family are predominantly positioned close to chromosome ends. Characteristically, these regions are transcriptionally inert and demarcated by the repressive histone mark H3K9me3 and the evolutionary conserved silencing factor *P. falciparum* heterochromatin protein 1 (PfHP1). It is believed that this specialised environment at chromosome ends generates a structural framework for the epigenetic control of *var* gene expression. Moreover, telomeres play a crucial role in preserving genome integrity by protecting chromosome ends from inappropriate fusion and recombination events, as well as in regulating telomere length.

However, we still lack a detailed functional understanding of the underlying molecular mechanisms that regulate *Plasmodium* chromosome end biology. During my PhD thesis, I tackled chromosome end biology from three different angles to improve our understanding of how virulence gene expression is regulated and how genome integrity is preserved.

In a first project I performed an in-depth functional analysis of the epigenetic silencing factor PfHP1 by generating an inducible loss-of-function mutant. We showed that upon PfHP1 depletion parasites display a complete breakdown of mutually exclusive *var* expression and antigenic variation. Intriguingly, we also found that over 50% of PfHP1-deprived parasites represented viable gametocytes that complete sexual development up to stage V maturity. This high conversion rate was linked to the targeted de-repression of the *ap2-g* locus that codes for the ApiAP2 transcription factor AP2-G, which is essential for gametocyte conversion. Thus, our data unveiled PfHP1 not only as a master regulator of variegated expression of exported virulence factors, but also as a crucial factor in the regulation of sexual cell differentiation.

In a second project I aimed at the functional characterisation of the chromosome-end associated protein PfSIP2, which was shown to specifically interact with SPE2 elements in subtelomeric regions. In-depth analysis of the expression profile of endogenous PfSIP2 revealed that this protein is only expressed during a very narrow time window of approximately 10hrs in late stage parasites, which coincides with intra-erythrocytic schizogony. Genome-wide ChIP-Seq experiments confirmed the exclusive binding of endogenous PfSIP2 to subtelomeric SPE2 landmarks in *ups*B *var* promoter regions and subtelomeric non-coding regions. Surprisingly, however, neither phenotypic changes nor differential gene expression were observed in a conditional PfSIP2-loss-of-function mutant and hence this approach didn't uncover novel insights into the function of this ApiAP2 factor.

In a third project I aimed at the identification of the telomere repeat-binding factor (TRF) in *P. falciparum*. Although TRFs are highly conserved and play essential roles in preserving chromosome integrity and regulating chromosome length in model eukaryotes, so far no TRF homologue has been found in the malaria parasites. My work reports about the successful *de novo* identification of the *P. falciparum* telomere repeat-binding protein (PfTRF). Intriguingly, this protein appears to be evolutionary distinct from TRFs in other eukaryotes as it binds to telomere repeat DNA via a C-terminal C_2H_2-type zinc finger domain instead of a MYB domain. Genome-wide mapping by ChIP-Seq experiments not only confirmed that PfTRF indeed binds to all chromosome termini *in vivo,* but as well revealed an unexpected second binding hotspot at telomere repeat-like sequences found in subtelomeric *var* gene promoters. A comprehensive characterisation of PfTRF using a conditional loss-of-function mutant identified essential roles for this protein in mitotic cell cycle progression and telomere length regulation. Hence, our findings provide important new insight into mechanisms underlying genome maintenance and possibly virulence gene silencing in *P. falciparum*. They further suggest that malaria parasites employ an evolutionary divergent molecular complex to preserve telomere function.

In summary, my results provide important new and detailed understanding of the molecular processes involved in genome maintenance, virulence gene expression and sexual conversion in *P. falciparum*, processes that are highly relevant for malaria pathogenesis, parasite viability and malaria transmission. I am confident that these findings have important implications for the development of intervention strategies targeting parasite propagation and transmission.

Chapter 1: General Introduction

Malaria still is the most preeminent and deadly parasitic disease in humans, predominantly affecting children below the age of five in sub-Saharan Africa. With nearly half of the world's population at risk and 600'000 malaria deaths every year, the disease represents not only a major health burden, but also imposes a huge drawback for social and economic development in endemic countries (WHO, 2014).

The apicomplexan malaria parasite is transmitted through the bites of infected *Anopheles* mosquitos. Amongst the five species infecting humans, *Plasmodium falciparum* is responsible for the most severe outcomes of malaria (malaria tropica), and hence contributes to the highest morbidity and mortality burden of total malaria infections (Jongwutiwes et al., 2004; Tuteja, 2007). One of the major reasons for the refractoriness of the parasite to intervention strategies is its extraordinarily complex life cycle, as well as its sophisticated strategies to avoid host immune responses.

The life cycle of *Plasmodium* parasites comprises multiple stages of asexual replication in the human host and the female mosquito vector *Anopheles*, as well as obligate sexual reproduction in the mosquito. After sporozoites are transmitted to the human host during a mosquito blood meal, they migrate to the liver to infect hepatocytes. After tremendous amplification by asexual replication, the intra-erythrocytic development cycle (IDC) is initiated through invasion of red blood cells (RBC) by merozoites. Here, the parasite again undergoes asexual replication by developing from so-called ring and trophozoite to schizont stages, which ultimately causes rupture of the infected erythrocytes and the release of up to 32 merozoites ready to reinvade new RBCs (Tuteja, 2007). While most of the parasites undergo repeated rounds of replication, a small proportion of these intra-erythrocytic forms differentiate into male and female gametocytes, initiating the sexual phase of the life cycle (Smith et al., 2000). After these sexual precursor cells are taken up during a mosquito blood meal, fertilization takes place and – after a complex cascade of further developmental steps – infectious sporozoites accumulate within the mosquito salivary glands, ready to be transmitted to the next human host (Menard et al., 2013; Tuteja, 2007).

1.1. Antigenic variation secures parasite survival and transmission

After erythrocyte invasion, extensive host cell remodelling processes facilitate the export of the major virulence factor *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) to the surface of the infected RBC. The interaction of PfEMP1 with various receptors (e.g. CD36 and ICAM-1) on host endothelial cells as well as with uninfected RBCs leads to parasite sequestration, which prevents phagocytic clearance in the spleen (Aikawa et al., 1990; Craig

and Scherf, 2001; Miller et al., 2013; Newbold et al., 1997; Reeder et al., 1999; Turner et al., 1994). This pathophysiological formation of erythrocyte aggregates can disrupt blood flow in the vasculature of vital organs and induce pro-inflammatory and coagulation responses in the human host, which is directly linked to the fatal outcome of malaria infections (Hviid and Jensen, 2015; Pasternak and Dzikowski, 2009).

Exposure of parasite-derived proteins on the RBC surface, however, also provides the immune system with a target that allows inducing antibody-mediated responses to fight the infection. In order to avoid elimination by the host immune system, the parasite employs clonally variant expression of PfEMP1. This major surface antigen is encoded by 60 primarily subtelomeric gene variants of the *var* gene family. Importantly, transcriptional activation of *var* genes is mutually exclusive (Gardner et al., 2002; Scherf et al., 1998, 2008). As a consequence, only a single member is active in each parasite at any time, while all others remain silenced by default. Switches in *var* gene activity, lead to antigenic variation of PfEMP1 and mutual exclusion/singular gene choice limits antigenic exposure to the host immune system. This is key for establishing long-term infection and ultimately securing transmission (Dzikowski et al., 2006a; Pasternak and Dzikowski, 2009; Scherf et al., 2008).

Clearly, the parasite makes use of sophisticated regulatory mechanisms to control its complex life cycle as well as to regulate the transcription of *var* genes, responsible for the chronic and fatal outcome of many *P. falciparum* infections. Research over the past years has made evident that this level of sophistication is achieved through epigenetic as well as transcriptional processes that, in turn, are influenced by the spatial organisation of chromosomal information.

1.2. Genome organisation of *P. falciparum*

A milestone towards understanding the complex biology of malaria parasites certainly was the success in completing the whole genome sequence of the *Plasmodium falciparum* parasite strain 3D7 in 2002 (Gardner et al., 2002). The 23Mb genome of *P. falciparum* is haploid and structured into 14 linear chromosomes ranging from 0.7Mb to 3.4Mb in size. A total of 5409 open reading frames (ORF) were identified, of which 60% showed no similarity to known genes of other organisms (Gardner et al., 2002). Moreover, with an average length of 2.3kb per functional gene unit, *Plasmodium* ORFs are about 50% longer than those found in any other eukaryotic organism studied so far (Gardner et al., 2002). Genome sequencing revealed an extreme bias in nucleotide composition towards adenine and thymine bases. In fact, with 80.2% the AT content in *P. falciparum* is higher than in any other organism. Whereas the AT content is relatively lower in telomere-proximal and gene-coding sequences (70%), it is higher in intergenic regions (90%) and reaches up to 97% at the centromere

(Gardner et al., 2002; Hoeijmakers et al., 2012, 2013; Iwanaga et al., 2010; Kelly et al., 2006).

Interestingly, even though the chromosomal length varies considerable between of the 14 chromosomes, the genomic distribution of genes along the chromosomes seems to follow a general pattern. Whereas highly conserved single-copy genes coding for housekeeping functions are distributed throughout the central core of each chromosome, a large number of highly polymorphic species-specific genes cluster to the telomere-proximal regions (Gardner et al., 2002; Hall et al., 2005). These genes primarily encode members of large and hypervariable protein families that are implicated in functions at the host-parasite interface, including the four most prominent families PfEMP1, RIFIN, STEVOR and PfMC-2TM. Intriguingly, a recent study that investigated the transcriptional profiles of isogenic clones showed that most of these gene classes are subject to clonally variant gene expression (CVGE) and are associated with a heterochromatic chromatin structure (Flueck et al., 2009; Lopez-Rubio et al., 2009; Rovira-Graells et al., 2012). CVGE represents a prerequisite for antigenic variation, which allows for rapid adaptation to changing environments and has emerged as the main survival strategy of blood stage parasites (Rovira-Graells et al., 2012; Voss et al., 2014). Hence, in order to understand mechanisms underlying antigenic variation, it is important to investigate and understand the unique structural and functional properties of chromosomal extremities in this parasite.

1.2.1. *P. falciparum* chromosome ends

The parasite's telomeres are dominated by a double-stranded repetitive DNA sequence of the degenerative motif TT(T/C)AGGG, which terminates in a G-rich single-stranded 3' overhang. This structure conforms to the typical organisation of telomeres in eukaryotes (Bottius et al., 1998; Figueiredo et al., 2000; Gardner et al., 2002; Scherf et al., 2001; Vernick and McCutchan, 1988). In *P. falciparum*, the number of telomeric repeats varies dramatically between different chromosomes, as well as between the two telomeres on the same chromosome. Moreover, there is a great interspecies variability in telomere size, ranging from 1.2kb in *P. falciparum*, up to 6.7kb in *P. vivax* (Figueiredo et al., 2002). Nevertheless, the mean length of each chromosome end is kept constant over many replication cycles (Bottius et al., 1998). This is accomplished by the action of a reverse transcriptase enzyme, termed telomerase (PfTERT) (Bottius et al., 1998). It was shown that by the use of an RNA template (TERC), TERT is able to base-pair with the G-rich overhang sequence and synthesise telomere repeats *de novo* at chromosome ends (Blackburn, 2005; Greider and Blackburn, 1985, 1987; Yu et al., 1990). Hence, as in other eukaryotes, PfTERT is able to compensate for the loss of genetic material during each replication cycle that may be caused due to incomplete replication by conventional DNA polymerase mechanisms, and is therefore

imperative to maintain genome integrity (Blackburn, 2005; Bottius et al., 1998; Figueiredo et al., 2005; Zhao et al., 2014).

In contrast to the highly conserved telomere sequence, the adjacent telomere-associated region (TAS) – composed of both noncoding and coding regions – varies greatly between *Plasmodium* species, indicating that these regions underwent rapid evolution (Figueiredo et al., 2000). In *P. falciparum* the 20-40kb TAS is composed of six different non-coding telomere-associated repetitive elements (TAREs 1-6). While the orientation and relative order of the six TARE blocks is conserved, the size and sequence varies greatly between individual chromosome ends (Figueiredo et al., 2000; Gardner et al., 2002; Scherf et al., 2001). The coding part of the TAS region begins directly downstream of TARE 6 and is characterised by up to three *var* gene variants interspersed by members of other multigene families such as *rif and stevor* (Gardner et al., 2002).

Fluorescence *in situ h*ybridisation (FISH) studies revealed that *P. falciparum* chromosome termini are not randomly distributed in the nucleus, but form clusters comprised of 4-7 ends that are tethered to the nuclear periphery (Freitas-Junior et al., 2000). It was shown that TAS play an important role in the establishment of this nuclear architecture, since chromosomes lacking the TAS sequences are delocalised from chromosome-end clusters (Figueiredo et al., 2002). It is believed that the spatial telomere organisation provides an excellent structural framework for recombination events to generate an unlimited diversity of the virulence gene repertoire localised to these regions (Figueiredo et al., 2002).

1.3. Chromatin and Epigenetic control mechanisms

The highly complex life cycle of *P. falciparum* includes rapid stage transitions in both the human host and the *Anopheles* vector. This demands for gene expression control mechanisms that allow the parasite to respond to the rapidly changing conditions encountered during each development stage. During the past years it became obvious that epigenetic mechanisms play a key role in this strategy.

The term epigenetics was first coined by C. H. Waddington in the year 1940. To date, it describes heritable chromatin states that allows for altering gene activity without changes in DNA sequence (Berger et al., 2009; Waddington, 2012). Nucleosomes represent the core unit of chromatin. They are composed of two copies of each of the histone proteins H2A, H2B, H3 and H4, enwrapping ~147bp of DNA. The nucleosomes are located side by side on the DNA strand exposing “free” so-called linker DNA between nucleosomal units (Kornberg and Lorch, 1999; Luger et al., 1997). Depending on the distance of nucleosome positioning, chromatin structure is generally divided into two major distinct states. In euchromatin, nucleosomes are arranged in a loose fashion and DNA is thus relatively accessible for DNA-

binding proteins. Euchromatin is the predominant structure in actively transcribed genes. By contrast, in heterochromatin, nucleosomes are located contiguous to each other, making DNA inaccessible to the transcription machinery (Jiang and Pugh, 2009).

The regulation of nucleosome positioning is crucial in regulating the access of enzymes that read, transcribe, replicate and repair DNA at the desired locations. One mechanism of how nucleosome organisation can be changed is through the replacement of core histones with histone variants. Compared to canonical histones, the structural differences of variants may have strong negative or positive effects on nucleosome stability and DNA compaction (Segal and Widom, 2009; Venkatesh and Workman, 2015). Further, nucleosome positioning may also be altered by specific post-translation modifications (PTM) of the flexible N-terminal tail of histones (Berger, 2002; Kouzarides, 2007). Histone N-tails, emanating from the nucleosome core complex, are among the most highly conserved sequences in eukaryotes. Covalent modifications include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, de-imination and proline isomerisation. Over 60 different residues on histones prone to modifications have been described so far, and diversity is further increased by the fact that modifications are conducted in up to three different forms (for example: mono-, di- and tri-methylation) (Berger, 2002; Kouzarides, 2007). This “histone code” is created by so-called code “writers” that catalyse the addition of modifications to the N-terminal histone tail through specialised domains harbouring enzymatic functions. Recognition of the histone-code by code “readers”, specifically binding to a certain histone modification, in turn allows for the recruitment of downstream effector proteins, ultimately condensing/loosening nucleosomal arrays (Strahl and Allis, 2000).

1.3.1. The chromatin landscape in *P. falciparum*

The genome of *P. falciparum* encodes single copies of each of the four core histones H4, H3, H2B and H2A, forming the core nucleosome complex (Trelle et al., 2009). The presence of four histone variants H2A.Z, H2B.Z, H3.3 and CenH3, together with the complex mix of over 50 histone tail modifications as well as the rich complement of code “writer” and “reader” proteins, suggests that epigenetic transcriptional control is an important constituent of the gene expression logic in *Plasmodium* parasites (Cary et al., 1994; Talbert et al., 2012).

Euchromatin in chromosome internal regions harbours housekeeping genes that are actively transcribed during the IDC of blood stage parasites. This transcriptionally permissive state is hallmarked by the presence of acetylated lysine 9 of histone H3 (H3K9ac) and tri-methylated lysine 4 of histone 3 (H3K4me3) (Bártfai et al., 2010; Karmodiya et al., 2015; Lopez-Rubio et al., 2009; Salcedo-Amaya et al., 2009; Trelle et al., 2009). The presence of these activating histone modifications in promoter regions abolishes the positive charge of nucleosomes.

Consequently, nucleosomes loose contact with DNA, which in turn allows access for the transcriptional machinery. Moreover, acetylation of histone marks can facilitate transcription by recruiting activating effector proteins (Grunstein, 1990, 1997). Moreover, euchromatic intergenic regions are hallmarked by the presence of a special histone double-variant containing both, the histone variant H2A.Z and the apicomplexan-specific histone variant H2B.Z (Bártfai et al., 2010; Hoeijmakers et al., 2013; Petter et al., 2013; Talbert et al., 2012). It is believed that replacement of canonical histones by this double-variant nucleosome reduces nucleosome stability, which consequently allows for generating a transcriptionally-permissive environment at the transcriptional start site and hence regulates transcriptional activation of genes (Hoeijmakers et al., 2013).

In contrast, 10% of the *P. falciparum* genome resides in a transcriptionally-repressive heterochromatic state and is demarcated by the presence of tri-methylated lysine 9 of histone 3 (H3K9me3) and the *P. falciparum* heterochromatin protein 1 (PfHP1) (Flueck et al., 2009; Lopez-Rubio et al., 2009; Pérez-Toledo et al., 2009; Salcedo-Amaya et al., 2009). The histone H3K9me3 modification is a hallmark for epigenetic gene silencing, serving as a docking station for the HP1 protein to mediate chromatin compaction (Krauss, 2008). Strikingly, these heterochromatic domains almost exclusively harbour gene families that are involved in antigenic variation, including the *var* family (Rovira-Graells et al., 2012; Voss et al., 2014).

As explained above, *var* genes are subject to mutually exclusive transcription, where only one single variant is actively transcribed while the other ~60 variants remain silenced (Kyes et al., 2007b; Scherf et al., 1998). Intriguingly, whereas the H3K9me3 mark covers all silenced *var* genes, the sole active family member is devoid of this histone mark and is marked by the activating histone modifications H3K9ac and H3K4me2/3 as well as by the histone variant H2A.Z instead (Chookajorn et al., 2007; Hoeijmakers et al., 2013; Lopez-Rubio et al., 2007; Petter et al., 2011). It is believed that H3K4me2/3 modifications provide a heritable mark for the active *var* gene, which allows bookmarking it during non-transcribed phases of the IDC for the expression in the subsequent generation (Lopez-Rubio et al., 2007). Work by Volz and colleagues suggested that the histone methyltransferase PfSET10 plays a major role in positioning this histone mark at the active *var* loci (Volz et al., 2012). Recently, Jiang and colleagues showed that active *var* transcription is also characterised by reduced levels of H3K36me3, particular at the transcriptional start site and the intron region. Deposition of this histone mark at silenced *var* genes depends on the methyltransferase PfSET2. Consistent with these findings, a PfSET2 knockout cell line resulted in the simultaneous de-repression of all *var* gene variants (Jiang et al., 2013). It is proposed that the specific recruitment of PfSET2 to *var* loci is accomplished through its tethering to

unphosphorylated RNA Polymerase II (RNAPII), potentially during the production of non-coding RNAs (Ukaegbu et al., 2014).

Taken together, these findings suggest that *var* gene silencing and activation is at least partially orchestrated through the presence and absence of the histone marks H3K9me3/H3K36me3 or H3K9ac/H3K4me3/2, respectively.

Interestingly, several research groups showed that the NAD+-dependent class III histone deacetylase (HDAC) silent information regulator 2 proteins, PfSir2A and PfSir2B, are involved in generating/maintaining a repressive state in subtelomeric regions by removing histone acetylation marks (Duraisingh et al., 2005; Freitas-Junior et al., 2005; French et al., 2008; Merrick et al., 2010; Tonkin et al., 2009). Histone hypoacetylation is central for heterochromatin formation and is hence believed to provide an epigenetic framework for the silencing of *var* gene variants. Indeed, loss of PfSir2A and PfSir2B knockout cell lines results in the simultaneous de-repression of numerous silenced *var* genes (Duraisingh et al., 2005; Merrick et al., 2010; Tonkin et al., 2009). More recently, Coleman and colleagues showed that another histone deacetylase, PfHda2, is also essential for global *var* gene silencing (Coleman et al., 2014).

1.3.2. The Heterochromatin protein 1 in *P. falciparum*

In other eukaryotes, it was shown that the histone mark H3K9me3 serves as a docking site for the specific recruitment of HP1 proteins (Lachner et al., 2001; Nakayama et al., 2001). HP1 plays critical roles in a variety of chromatin-related processes such as epigenetic silencing, DNA replication, sister chromatid cohesion and genome stability (Hediger and Gasser, 2006; Hiragami and Festenstein, 2005; So Hee Kwon and Jerry L. Workman, 2008; Zeng et al., 2010). These multifaceted functions of HP1 are guided by its N-terminal chromodomain (CD) that specifically binds to the H3K9me3 mark as well as the C-terminal chromoshadow domain (CSD) that mediates both protein homo- and heterodimerisation (Lomberk et al., 2006). Through the recruitment of a H3K9-specific methyltransferase, neighbouring nucleosome receive new methylation marks and heterochromatin is able to spread over nucleosomal arrays in a self-perpetuating manner (Fritsch et al., 2010; Li et al., 2002; Lomberk et al., 2006).

The genome of *P. falciparum* encodes a single HP1 ortholog (PfHP1), which binds specifically to H3K9me3 marks (Flueck et al., 2009; Pérez-Toledo et al., 2009). In these studies, several attempts to generate a PfHP1 knockout parasite line failed suggesting an essential role for this protein for parasite survival. Genome-wide chromatin immuno-precipitation (ChIP) revealed a defined association of PfHP1 with 425 genes in subtelomeric and some chromosome-internal islands. Intriguingly, almost all of these genes represent

members of species-specific multigene families encoding virulence factors that are involved in host-parasite interactions, including all *var* genes. In addition, PfHP1 occupies a few specific loci located within otherwise euchromatic chromosomal regions (Flueck et al., 2009). This includes the locus coding for the ApiAP2 transcription factor AP2-G that is essential for gametocyte conversion in *P. falciparum* and *P. berghei* (Kafsack et al., 2014; Sinha et al., 2014).

1.3.3. Epigenetic control mechanisms at parasite telomeres

Interestingly, micrococcal nuclease digestion assays show that, whereas internal regions of *P. falciparum* telomeres are associated with nucleosomes, the outermost part of telomeres is organised into a non-nucleosomal chromatin structure (Figueiredo et al., 2000). In other eukaryotes, it is well established that this terminal part of the chromosome is bound by telomere-specific proteins to form a multimeric telosome/shelterin complex that serves multiple essential functions (Xin et al., 2008). Not only does it protect chromosomes by distinguishing their ends from DNA double-strand breaks, thereby preventing chromosome fusion and/or recombination events, but it also participates in chromosome length regulation and anchoring of telomeres to the nuclear periphery (Cech, 2004; Giardini et al., 2014; McEachern et al., 2000).

Without exception, eukaryotic telosome complex formation is initiated by telomere repeat-binding proteins (TRFs) that bind to double-stranded (ds) telomere repeat sequences via a MYB/MYB-like DNA-binding domain, followed by the recruitment of other telosome/shelterin components and additional interacting factors (Linger and Price, 2009). In *Saccharomyces cerevisiae*, this comprises histone-tail modifying enzymes, including the full Sir complex, composed of Sir2, Sir3 and Sir4. The histone deacetylase activity of this Sir complex is essential for the spread of the heterochromatic state into neighbouring coding regions (Kueng et al., 2013; Ottaviani et al., 2008). In *Schizosaccharomyces pombe*, the telosome complex recruits the H3K9-specific methyltransferase Clr4. Activity of this enzyme leads to the recruitment of the HP1 ortholog Swi6 that mediates chromatin compaction beyond telomeric regions (Kanoh et al., 2005; Nakayama et al., 2001). In both cases, this leads to the position-dependent silencing of genes located close to telomeric regions, a phenomenon collectively referred to as telomere position effect (TPE) (Gottschling et al., 1990).

In *P. falciparum*, the close proximity of the *var* family to chromosome ends exposes them to the dominant regulatory impact of the TPE (Duraisingh et al., 2005; Freitas-Junior et al., 2005; Mancio-Silva et al., 2008; Tonkin et al., 2009). However, despite the similarity in structure of *P. falciparum* telomeres compared to other eukaryotes, and the fact that TPE is

functional in *P. falciparum,* a TRF ortholog has not been identified and appears to be absent in these parasites.

However, similar to findings in model eukaryotes, PfHP1 was shown to be a major constituent of subtelomeric regions and hence a major role in TPE and *var* gene regulation is suggested for this protein. Apart from that, *P. falciparum* origin recognition complex subunit 1 (PfOrc1) and PfSir2A closely resemble *S. cerevisiae* Sir3 and Sir2, respectively (Mancio-Silva et al., 2008). Indeed, these proteins were shown to associate with telomeres and TAS (Deshmukh et al., 2012; Freitas-Junior et al., 2005; Mancio-Silva et al., 2008). Preliminary results further indicate that the N-terminal domain of PfOrc1 is involved in *var* gene silencing, possibly by facilitating heterochromatin formation through recruitment of PfSir2A (Deshmukh et al., 2012; Mancio-Silva et al., 2008). Further, it was also shown that a member of the Alba protein family, PfAlba3, exclusively localises to telomeric and subtelomeric regions. However, a potential role of PfAlba3 in TPE remains elusive to date (Goyal et al., 2012).

Clearly, the identification and characterisation of PfTRF and other telosome proteins will be key for gaining knowledge on how genome integrity is maintained and subtelomeric heterochromatin is established and regulated in *Plasmodium* spp.

1.4. Transcriptional regulation of the *var* multigene family

The involvement of epigenetic processes in the control of mutually exclusive *var* gene transcription is indisputable. While epigenetic factors that recruit and organise *P. falciparum* heterochromatin remain largely unknown, it became obvious that *var* promoter sequences provide sufficient information for epigenetic silencing and mutually exclusive transcription of this virulence gene family (Dzikowski et al., 2006b; Voss et al., 2007, 2006). According to sequence similarities in their 5' upstream (*ups*) regions, *var* promoters are grouped into three major types, termed *ups*A, *ups*B and *ups*C. This classification strongly correlates with the specific chromosomal location of *var* genes and their orientation of transcription. Whereas *var* genes located in internal regions of the chromosome are associated with *ups*C sequences, genes positioned at the subtelomeric region are associated with either *ups*B type promoters (if transcribed towards the centromere) or with *ups*A promoter elements (if transcribed towards telomeres) (Gardner et al., 2002; Kyes et al., 2007b; Lavstsen et al., 2003).

Whereas every *var* gene locus naturally adopts a silent state, they remain capable of *in situ* activation at the level of transcription initiation of RNA Polymerase II (RNAPII) (Kyes et al., 2007a; Scherf et al., 1998; Voss et al., 2006). In search for *cis*-acting elements regulating silencing of promoter sequences, two highly conserved motifs in the *ups*B promoter sequence (SPE1 and SPE2) and one highly conserved motif in the *ups*C promoter region

(CPE) were identified (Voss et al., 2003). It was shown that these three elements interact with distinct *trans*-acting DNA-binding proteins in a sequence-dependent manner. Interestingly, expression of these proteins coincides with *var* gene repression and transient transfection experiments indicate a direct participation of the respective DNA-binding activities in *var* gene regulation. In addition, targeting SPE2 motifs to a heterologous promoter caused alterations in nucleosomal organisation that coincided with transcriptional repression (Voss et al., 2007). Apart from *ups*B *var* promoters, SPE2 elements are also found in TARE2/3 region (Flueck et al., 2010) and are transcribed into long non-coding RNA (lncRNA) that have a potential role in nucleation of heterochromatin (Broadbent et al., 2011). Together with the resistance of SPE2 arrays to nuclease digestion, these finding suggest that SPE2 elements may be involved in the recruitment and organisation of heterochromatin in subtelomeric regions, eventually repressing *var* transcription (Voss et al., 2007). Recently, another transfection-based functional promoter mapping approach identified an autonomous upstream activation sequence (UAS) in *var* promoters. In addition, the same study mapped a 47bp *cis*-acting sequence element in the *var* 5' upstream region (MEE), which is central for incorporation of the *var* locus into the programme of mutually exclusive transcription (Brancucci et al., 2012).

Further efforts directed towards the understanding of *var* gene silencing led to the discovery of the *var* intron as another regulatory non-coding element. Specifically, it was shown that silencing of *var* genes involves the cooperative interaction between the *var* intron and the *var* upstream promoter elements (Calderwood et al., 2003; Deitsch et al., 2001; Gannoun-Zaki et al., 2005). Follow-up studies reinforced that there is a strict one-to-one pairing requirement between *var* promoters and introns for *var* gene silencing to occur (Dzikowski et al., 2007; Frank et al., 2006; Swamy et al., 2011). Consequently, unpairing of an upstream promoter from its adjacent intron, renders the locus constitutively active and unrecognised by the mechanism controlling mutually exclusive *var* expression (Dzikowski et al., 2007; Frank et al., 2006). Recently, the Dzikowski laboratory mapped a protein-binding TG-rich DNA element that is present in both *var ups* regions and *var* introns, and found this element to be essential in mediating promoter pairing between the intronic and upstream regulatory regions (Avraham et al., 2012). Further, it was shown that once this promoter-intron pair is established, it is maintained for many generations, suggesting an important role for these interactions in epigenetic memory (Swamy et al., 2011).

Moreover, it was shown that the function of the intron as a regulator of *var* gene expression is dependent on its own bi-directional promoter activity, which produces *var*-associated sense and antisense non-coding transcripts (Calderwood et al., 2003; Gannoun-Zaki et al., 2005). On the one hand, it was shown that the sense non-coding transcript localises to perinuclear areas and associates with chromatin and hence is proposed to play a role in

chromatin assembly and concomitant transcriptional silencing of the *var* gene family (Epp et al., 2009). On the other hand it was shown that the intronic antisense non-coding transcripts specifically associates with the single active *var* gene in ring stage parasites and plays a key role in regulating *var* gene activation (Amit-Avraham et al., 2015).

1.5. Transcriptional control and specific transcription factors

The IDC of *P. falciparum* underlies rigid timing and therefore requires tight transcriptional control (Bozdech et al., 2003; Le Roch et al., 2003). Like in all eukaryotes, transcription in *P. falciparum* is initiated through a pre-initiation complex composed of basal transcription factors guiding correct association of the RNA polymerase II (RNAPII) complex with the core promoter region. Subsequent binding of specific transcription factors allows for enhancing or repressing the transcriptional activity of RNAPII (Roeder, 2003).

Some progress has been made in describing *cis*-acting regulatory motifs in *Plasmodium* promoter regions and their cognate *trans*-acting specific transcription factors and elucidating their function in driving stage-specific gene expression (Horrocks et al., 2009; Painter et al., 2011). However, in contrast to epigenetic and chromatin structure regulators, the genome of *P. falciparum* reveals an unexpected paucity of specific transcription factors found in other eukaryotes (Callebaut et al., 2005; Coulson and Ouzounis, 2003; Coulson et al., 2004). In fact, there is a complete lack of proteins carrying Hoemo, bZip, bHLH or FkH domains, all of which are found in conserved transcription factor families in other eukaryotes (Aravind et al., 2003; Coulson et al., 2004). Until recently, only very few representatives carrying C_2H_2-type zinc-finger domains or E2F domains have been identified, and most of them show sequence homology to basal transcription factors, rather than specific transcription factors (Anantharaman et al., 2007; Aravind et al., 2003; Iyer et al., 2008; Templeton et al., 2004). This is also true for the TATA-binding protein (PfTBP) (Horrocks et al., 2009; McAndrew et al., 1993; Ruvalcaba-Salazar et al., 2005).

Recent computational analysis of DNA-binding domains in Apicomplexans revealed a new family of transcription factors, termed ApiAP2 that is related to the Apetala2 (AP2) domain protein family in plants (Balaji et al., 2005). Each member is characterised by at least one copy of an AP2-like DNA-binding domain and most of them show stage-specific expression during the IDC (Balaji et al., 2005; Bozdech et al., 2003; Le Roch et al., 2003). The ApiAP2 domains of the 27 family members identified in *P. falciparum* show sequence-specific DNA preferences and are thus excellent candidate regulators for the coordinated cascade of gene expression along the *P. falciparum* life cycle (Campbell et al., 2010, 2010). Indeed, ApiAP2 protein were identified as key players not only during the IDC, but also in the regulation of parasite development in liver stages, ookinetes, sporozoites and gametocytes (Iwanaga et

al., 2012; Kafsack et al., 2014; Kaneko et al., 2015; Sinha et al., 2014; Yuda et al., 2009, 2010).

One particular ApiAP2 factor, termed SPE2-interacting protein (PfSIP2), was shown to specifically interact with SPE2 elements located upstream of *ups*B-type *var* genes as well as within the telomere-associated repetitive elements 2 and 3 (TARE2/3) (Flueck et al., 2010). Since several attempts to knock out PfSIP2 failed, an essential role for this factor in parasite survival is suggested. Consistent with its exclusive location to subtelomeric regions, overexpression of the protein had no effect on global gene transcription. Instead, a role for PfSIP2 in several other processes including chromosome replication and/or segregation and/or the nucleation of subtelomeric heterochromatin has been proposed (Flueck et al., 2010).

To date, only two other transcription factors outside the AipAP2 family have been investigated in *Plasmodium*. One of them, the Myb1 homolog PfMyb1 (PF3D7_1315800), was reported to bind specifically to several promoter elements via a Myb-like domain and to directly regulate key genes involved in cell cycle regulation and progression (Gissot et al., 2005). Further, the KH-domain containing protein PREBP (PF3D7_1011800) has recently been described as a novel and unique transcription factor in *Plasmodium* species, able to enhance transcriptional activity of a target promoter carrying a 102bp Prx Regulatory Element (PRX) (Komaki-Yasuda et al., 2013).

1.6. Overall Rationale and Objectives

The specialised biology of chromosome ends generates the overall structural and functional framework for epigenetic control of subtelomeric gene expression, and this has emerged as the major survival strategy of *P. falciparum* parasites during blood stream infection in the human host. However, our knowledge in this field is still rather descriptive as we lack a detailed functional understanding of the underlying molecular mechanisms. Hence, the overall objective of my research was to identify novel regulators, as well as to functionally investigate known components of the epigenetic machinery at chromosome ends, in order to improve our understanding of how subtelomeric heterochromatin is established, maintained and contributes to phenotypic variation in *P. falciparum*.

More specifically, I aimed at the functional characterisation of heterochromatin protein 1 PfHP1 (Chapter 2; Project 1) and the SPE2-interacting protein PfSIP2 (Chapter 3; Project 2) by addressing their role in virulence gene regulation as well as in heterochromatin formation. In a third project, I aimed at the identification and characterisation of the unknown *P. falciparum* telomere repeat-binding factor PfTRF (Chapter 4, Project 3).

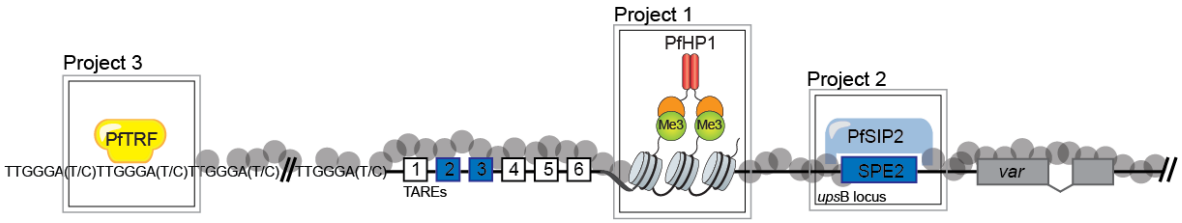

Figure 1. Summary of the research objectives to explore regulators of chromosome end biology and virulence gene expression in *P. falciparum*

Schematic representation of *P. falciparum* chromosome ends that are composed of the DNA repeat sequence elements TT(T/C)AGGG, neighbouring telomere-associated repetitive elements TARE1-6, and adjacent *var* genes and members of other subtelomeric gene families. The entire region is heterochromatic marked by H3K9me3/PfHP1 (grey circles). PfSIP2 protein binds to SPE2 arrays localised in *ups*B promoter regions and in TARE2/3. Ds telomere repeat sequences are proposed to interact with an unknown telomere repeat-binding factor PfTRF. PfHP1, PfSIP2 and PfTRF are believed to regulate heterochromatin formation, providing a structural framework for virulence gene expression and genome maintenance. **Project 1:** Functional analysis of PfHP1. **Project 2:** Functional characterisation of PfSIP2. **Project 3:** Identification and subsequent functional characterisation of the *P. falciparum* telomere repeat-binding factor PfTRF.

1.7. References

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Chapter 2: Heterochromatin protein 1 secures survival and transmission of malaria parasites

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I generated the clonal parasite line and I performed Western Blot analysis of PfHP1 expression levels (Fig. 1C), as well as microcalorimetry analysis (Fig. 1E). Further, I analysed sexual conversion rates (Fig. 2), as well as performed Flow Cytometry analysis (Fig. 3A). I performed rescue experiments of cell cycle arrested parasites (Fig. 3B-D&S5) and generated the parasitic material for microarray analysis. Moreover, I performed anti-ATS Western blots and IFAs (Fig. 6B&C), as well as rescue experiments to analyse gametocyte differentiation (Fig. 7B). Furthermore, I performed anti-H3K9me3 ChIP-qPCR experiments (Figure S6) and generated parasitic material to confirm induction of novel gametocyte genes (Fig. S7). I analysed data throughout the entire study, prepared illustrations and wrote the manuscript.

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2.1 Abstract

Clonally variant expression of surface antigens allows the malaria parasite *Plasmodium falciparum* to evade immune recognition during blood stage infection and to secure malaria transmission. Here, we demonstrate that conditional depletion of heterochromatin protein 1 (HP1), an evolutionary conserved regulator of heritable gene silencing, de-represses hundreds of heterochromatic virulence genes and disrupts the elusive mechanism underlying mutually exclusive expression and antigenic variation of the major virulence factor PfEMP1. Remarkably, we also discovered that the PfHP1-dependent regulation of PfAP2-G, a transcription factor required for gametocyte conversion, controls the switch from asexual parasite proliferation to sexual differentiation. This provides important new insight into the mechanism underlying gametocyte conversion and establishes a new concept of HP1-dependent cell differentiation in unicellular eukaryotes. Our study shows that PfHP1-mediated clonally variant gene expression is of fundamental importance for parasite survival and transmission and has major implications for developing novel anti-disease and transmission-blocking interventions against malaria.

2.2 Highlights

- Sexual commitment of malaria parasites is epigenetically regulated by PfHP1
- PfHP1 depletion disrupts singular *var* gene choice in *Plasmodium falciparum*
- PfHP1 is essential for mitotic proliferation of malaria blood stage parasites

2.3 Introduction

The protozoan parasite *Plasmodium falciparum* elicits the most severe form of malaria in humans and causes several hundred million clinical cases and 700,000 deaths annually (World Health Organisation, 2013). Malaria morbidity and mortality occurs due to the massive expansion of the parasite population during blood stage infection. Here, parasites mature intracellularly through the ring and trophozoite stages, before successive S-/M-phases produce a multinucleated schizont that releases up to 32 merozoites ready to invade new red blood cells (RBCs). In order to secure survival and establish chronic blood stage infection, *P. falciparum* employs clonally variant gene expression (CVGE) as a means to adapt to environmental challenges in the human host, in particular those imposed by the immune system (Rovira-Graells et al., 2012; Cortes et al., 2012).

The most striking example of CVGE is erythrocyte membrane protein 1 (PfEMP1), the major antigen and prime immune target on the surface of infected RBCs (iRBCs) (Scherf et al.,

2008). PfEMP1 is encoded by the 60-member *var* gene family (Su et al., 1995; Baruch et al., 1995) and mediates cytoadherence of iRBCs to microvascular endothelium, which prevents parasite clearance in the spleen and causes pathology that contributes substantially to severe malaria outcomes (Kyes et al., 2001). *var* transcription conforms to the concept of singular gene choice (or mutual exclusion); in each parasite only a single *var* gene is active while all other members remain silenced (Scherf et al., 1998). Transcriptional switches in *var* gene expression result in CVGE and consequently antigenic variation of PfEMP1 and immune evasion (Smith et al., 1995; Scherf et al., 1998). Importantly, this survival strategy is directly linked to malaria transmission; during each replicative cycle a small number of parasites commit to sexual development and differentiate into mature stage V gametocytes, the only stages capable of transmitting the infection to the mosquito vector (Baker, 2010).

Singular *var* gene choice is regulated by a poorly understood interplay between transcriptional and epigenetic control mechanisms (Guizetti and Scherf, 2013). Particularly striking is the observation that *var* genes are associated with histone 3 lysine 9 trimethylation (H3K9me3) and heterochromatin protein 1 (HP1) (Salcedo-Amaya et al., 2009; Lopez-Rubio et al., 2009; Flueck et al., 2009; Perez-Toledo et al., 2009; Chookajorn et al., 2007; Lopez-Rubio et al., 2007). HP1 is an evolutionary conserved regulator of heterochromatin formation and heritable gene silencing and was originally described in *Drosophila melanogaster* as a suppressor of position effect variegation (Eissenberg et al., 1990). HP1 binds to H3K9me2/3, the hallmark histone modification of heterochromatin, and recruits H3K9-specific methyltransferases (HKMTs) that modify adjacent nucleosomes (Lomberk et al., 2006). As a result, HP1 sustains a self-perpetuating mechanism for heterochromatin spreading and heritable gene silencing. In addition, HP1 also regulates euchromatic genes and is involved in other chromatin-related processes including cohesion, telomere maintenance, or DNA replication and repair (Kwon and Workman, 2008). This functional versatility is linked to the evolution of HP1 paralogs, particularly in metazoans, and the ability of HP1 to recruit functionally diverse proteins (Lomberk et al., 2006; Kwon and Workman, 2008).

P. falciparum contains only a single HP1 protein that localises primarily to H3K9me3-enriched heterochromatic regions. These chromosomal domains incorporate all *var* genes and hundreds of other clonally variant genes (such as *rifin*, *stevor* and *pfmc-2tm*) encoding species-specific blood stage antigens. At the same time, PfHP1 is also found at a small number of euchromatic loci (Flueck et al., 2009; Perez-Toledo et al., 2009). PfHP1 over-expression leads to increased silencing of some heterochromatic genes (Flueck et al., 2009) and the presence or absence of PfHP1 is linked to the silenced or active state of *var* genes, respectively (Perez-Toledo et al., 2009). Together, these observations suggest key functions for PfHP1 in heritable silencing and phenotypic variation of a large set of factors implicated in host-parasite interactions and immune evasion. However, if and to what extent PfHP1 is

indeed required for mutually exclusive *var* expression and/or for CVGE in general is unknown. Moreover, since the *pfhp1* locus is refractory to genetic deletion (Flueck et al., 2009; Perez-Toledo et al., 2009), additional unknown HP1-dependent pathways essential for parasite proliferation are likely to exist in *P. falciparum.*

Here, we conducted a comprehensive functional analysis of PfHP1 by generating a conditional PfHP1 loss-of-function mutant. We show that PfHP1 is indispensable for the heritable silencing of heterochromatic genes in general and in particular for the maintenance of singular *var* gene choice and antigenic variation of PfEMP1. In addition, PfHP1 is required at the G1/S transition phase for mitotic proliferation of blood stage parasites. Intriguingly, we also discovered that PfHP1 controls sexual commitment by regulating the bistable expression of single euchromatic locus encoding an ApiAP2 transcription factor.

2.4 Results

2.4.1 PfHP1 is indispensable for mitotic proliferation of blood stage parasites

We applied the FKBP destabilisation domain (DD) technique that allows modulating expression levels through the stabilising compound Shield-1 (Banaszynski et al., 2006; Armstrong and Goldberg, 2007), and generated a clonal parasite line expressing endogenous PfHP1 as a C-terminally tagged GFP-DD fusion (3D7/HP1^{ON}) (Figure S1). In presence of Shield-1, 3D7/HP1^{ON} parasites exhibited no growth phenotype (Figure 1A) and multiplied at a similar rate within a single asexual replication cycle (3.8-fold +/-0.6 SD) as 3D7/HP1^{ctrl} parasites in which PfHP1 is tagged with GFP only (4.4-fold +/-0.4 SD). When Shield-1 was withdrawn at 4-12 hours post-invasion (hpi), 3D7/HP1^{OFF} parasites completed the current intra-erythrocytic developmental cycle (IDC) and subsequent ring stage development with normal kinetics. Strikingly, however, these parasites arrested prior to schizogony in generation 2 (Figure 1A) and all efforts to select for proliferating subpopulations were unsuccessful.

Live cell imaging revealed the expected perinuclear localisation of tagged PfHP1 in 3D7/HP1^{ON} and 3D7/HP1^{ctrl} parasites throughout the IDC, whereas in 3D7/HP1^{OFF} parasites PfHP1 was undetectable 12 hours after Shield-1 withdrawal (Figure 1B). A more direct assessment by parallel Western blot and immunofluorescence assays (IFA) showed that after Shield-1 removal at 4-12hpi, PfHP1 was still detectable but reduced in late ring stages (16-24hpi) and early schizonts (32-40hpi) and localised diffusely to the nucleo- and cytoplasm (Figure 1C). After re-invasion, PfHP1 was undetectable in 3D7/HP1^{OFF} parasites by both methods. Similarly, targeted chromatin immunoprecipitation (ChIP-qPCR) showed that PfHP1-occupancy at subtelomeric (PF3D7_0426000) and chromosome-internal

(PF3D7_0412400) *var* loci was unchanged in late ring stages but substantially reduced in schizonts and subsequent generation 2 ring stages (Figure 1D).

We next analysed parasite viability using isothermal microcalorimetry (Wenzler et al., 2012). In generation 1, 3D7/HP1^{ON} and 3D7/HP1^{OFF} populations both displayed a typical heat emission profile marked by increased heat flow in trophozoites and schizonts (Figure 1E). In generation 2, however, the metabolic activity in PfHP1-depleted parasites changed dramatically and heat emission remained low over the entire 48 hour period of measurement. Importantly, these parasites were still viable since they emitted heat at a rate significantly higher compared to uninfected RBCs.

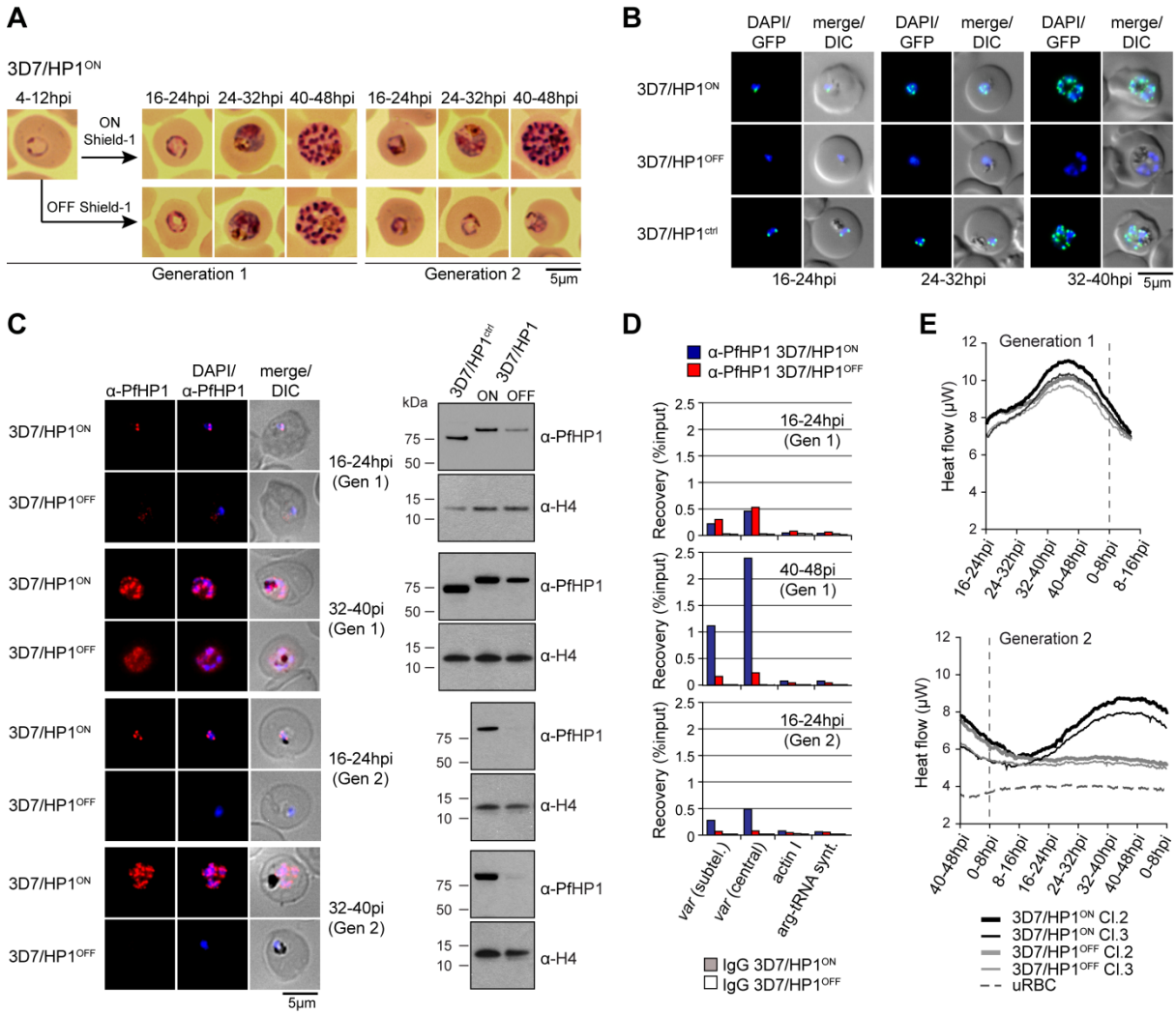

Figure 1. Growth phenotype of a conditional PfHP1 loss-of-function mutant and kinetics of PfHP1 depletion

(A) Giemsa-stained blood smears showing development of 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites over two generations (96hrs). See also Figure S1. (B) Expression and localisation of PfHP1 in 3D7/HP1^{ON}, 3D7/HP1^{OFF} and 3D7/HP1^{ctrl} parasites by live fluorescence microscopy (images taken 12 hours after removal of Shield-1). (C) Expression and localisation of PfHP1 in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites by IFA and Western blot (Shield-1 removal at 4-12hpi). The production and

specificity of affinity-purified polyclonal α-PfHP1 antibodies is described in Figure S1 and Supplemental Experimental Procedures. (D) PfHP1 occupancy at two heterochromatic *var* and two euchromatic control loci in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites was determined by ChIP-qPCR (Shield-1 removal at 4-12hpi). See also Figure S6. (E) Heat emission as determined by isothermal microcalorimetry in two 3D7/HP1 clones (Cl.2 and Cl.3) grown in presence or absence of Shield-1. uRBC, uninfected RBCs.

2.4.2 PfHP1 controls sexual differentiation

Intriguingly, prolonged microscopic observation revealed that PfHP1-depleted parasites consisted of a mixture of growth-arrested trophozoites and sexual forms undergoing gametocyte development (Figure 2A). Note that sexual conversion occurs through an unknown mechanism during the cell cycle prior to gametocyte development and that all daughter parasites released from a committed schizont undergo sexual differentiation (Bruce et al., 1990). To discriminate quantitatively between growth-arrested and sexual forms, we visualised the gametocyte-specific marker Pfs16 (Bruce et al., 1994) and knob-associated histidine-rich protein (KAHRP) (a marker for iRBCs) (Taylor et al., 1987) by indirect IFA.

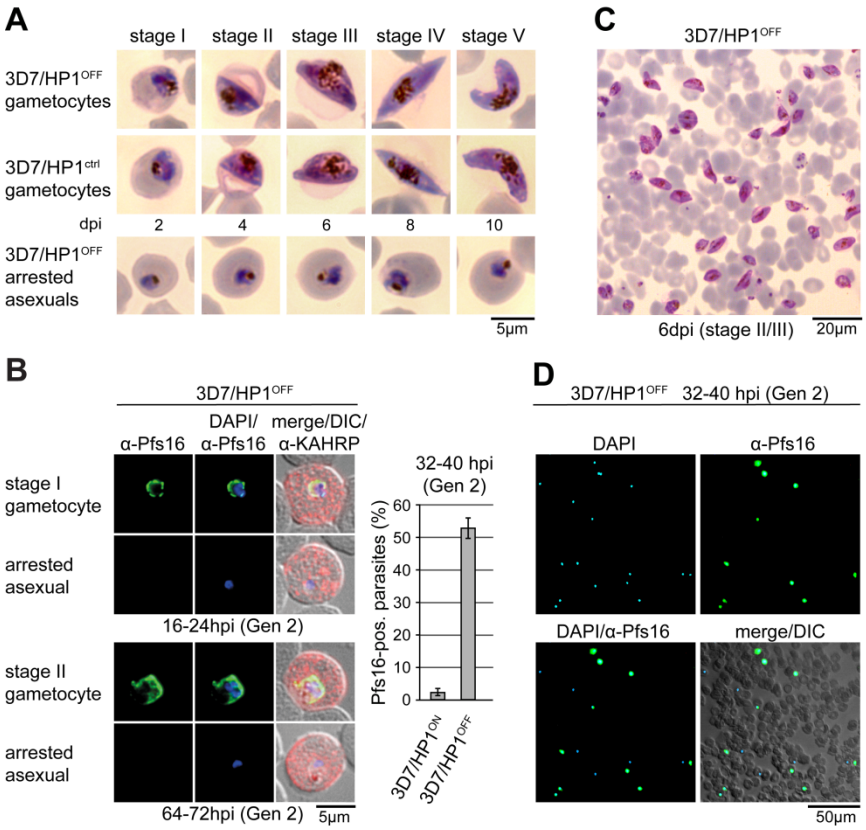

Figure 2. PfHP1 depletion induces gametocyte conversion

(A) 3D7/HP1^{OFF} and 3D7/HP1^{ctrl} gametocytes and cell cycle-arrested 3D7/HP1^{OFF} trophozoites. dpi, days post re-invasion. (B) Distinction between 3D7/HP1^{OFF} early gametocytes and arrested trophozoites by IFA (left) and proportion of Pfs16-/KAHRP-positive parasites in 3D7/HP1^{ON} and 3D7/HP1^{OFF} (right). Values show the mean (+/- SD) of three biological replicates (100 KAHRP-pos. iRBCs were scored per experiment). (C) Giemsa-stained blood smear of a 3D7/HP1^{OFF} parasite

culture (Shield-1 removal at 4-12hpi) at six days post re-invasion (dpi) (image taken at 60x magnification). The gametocyte hyper-induction phenotype is highlighted by the high proportion of stage II/III gametocytes among all iRBCs. (D) α-Pfs16 IFA of a 3D7/HP1^{OFF} parasite culture (Shield-1 removal at 4-12hpi) at 32-40 hours post re-invasion (image taken at 40x magnification). The gametocyte hyper-induction phenotype is highlighted by the high proportion of Pfs16-positive stage I gametocytes among all DAPI-positive iRBCs. See also Figure S2.

Remarkably, 52.7% (+/-3.1 SD) of 3D7/HP1^{OFF} parasites expressed Pfs16 in generation 2, compared to only 2.3% (+/-1.2 SD) of background conversion in the 3D7/HP1^{ON} population (Figure 2B). Overview images of a Giemsa-stained blood smear (six days post-invasion) and an α-Pfs16 IFA experiment (32-40hpi) provide visual confirmation of this unprecedented phenotype showing a high proportion of stage II/III and stage I gametocytes, respectively, in 3D7/HP1^{OFF} parasites (Figures 2C and 2D). Notably, PfHP1-depleted gametocytes completed sexual development within 8-10 days similar to control gametocytes (Figure S2). Hence, PfHP1 depletion triggers the synchronous hyper-induction of viable gametocytes, which demonstrates for the first time that sexual commitment in malaria parasites is epigenetically regulated.

2.4.3 PfHP1-depleted asexual parasites enter a reversible cell cycle arrest

To investigate at which stage of the cell cycle the non-gametocyte subpopulation of 3D7/HP1^{OFF} parasites arrested we performed single cell DNA content analysis by flow cytometry. This revealed that in contrast to 3D7/HP1^{ON} parasites, virtually all parasites in the 3D7/HP1^{OFF} population failed to replicate their genome in generation 2 (Figure 3A). While this is expected for non-proliferative gametocytes, this result demonstrates that the population of asexual parasites arrested prior to or during the first S-phase of schizogony. Interestingly, this cell cycle defect was reversible since PfHP1-depleted trophozoites re-entered S-phase and mitotic proliferation when Shield-1 was added back to the culture medium (Figure 3B). Even after 12 days in absence of Shield-1, rescued trophozoites re-accumulated perinuclear PfHP1 and progressed through schizogony (Figure 3C). This was not due to a genetic reversion as rescued parasites entered developmental arrest and gametocyte hyper-conversion when Shield-1 was withdrawn for a second time (data not shown). With prolonged time in absence of Shield-1, however, the parasitaemia decreased and the time required for growth resumption after Shield-1 replenishment increased, showing that a subset of PfHP1-depleted parasites died over time (Figure 3B and 3D). Together, these data corroborate the essential function of PfHP1 in mitotic proliferation and show that a subset of PfHP1-depleted trophozoites remained in a state of dormancy capable of re-entering the cell cycle if PfHP1 expression was restored.

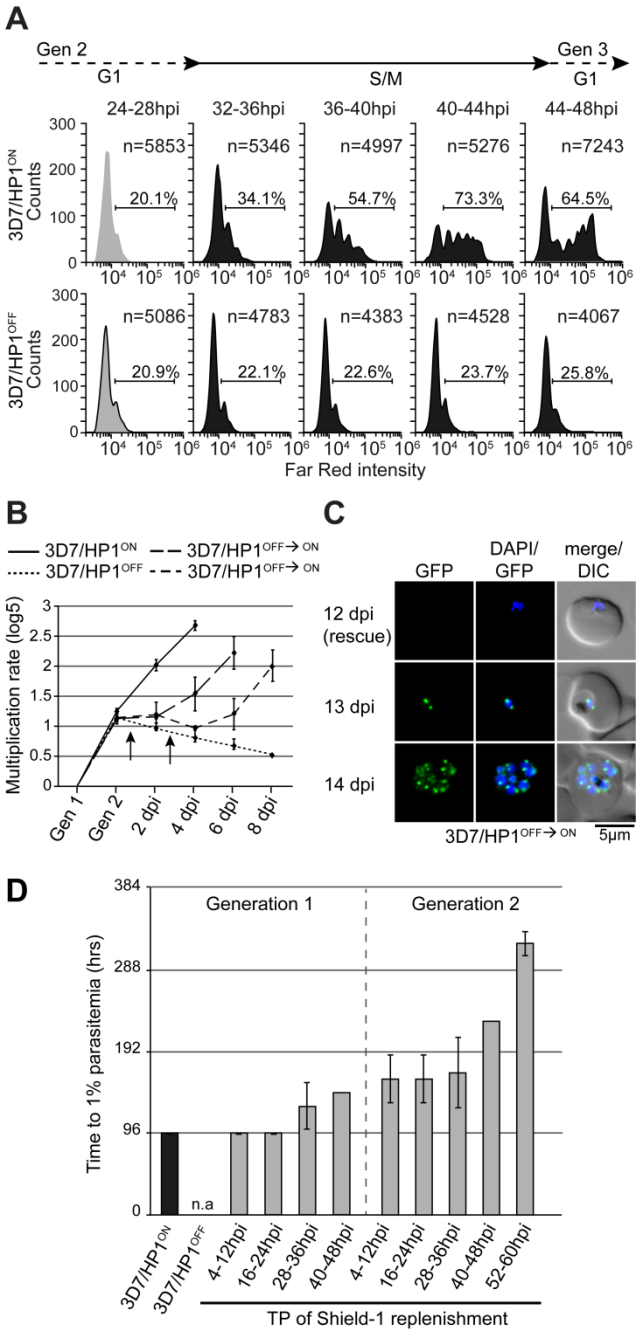

Figure 3. PfHP1 depletion causes reversible cell cycle arrest at the G1/S-transition phase

(A) Flow cytometry analysis of genomic DNA content in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites at five consecutive time points in generation 2. The percentage of iRBCs with ≥2 genomes is indicated. Prior to S-phase (grey) this value corresponds to RBCs infected with ≥2 parasites (confirmed by microscopy). *n*, number of gated iRBCs. (B) Cell cycle-arrested 3D7/HP1^{OFF} parasites re-establish asexual growth after adding back Shield-1 at 24 or 72 hours post re-invasion (arrows). Values show the mean of three biological replicates (+/- SD). (C) Growth-arrested 3D7/HP1^{OFF} parasites re-enter mitotic proliferation after Shield-1 replenishment. dpi, days post re-invasion. (D) Synchronous 3D7/HP1^{ON} cultures (~0.1% parasitaemia) were split at 0-8hpi and cultured either in presence or absence of Shield-1. Shield-1 was added back to 3D7/HP1^{OFF} cultures at nine consecutive TPs. Cultures were smeared daily and analysed by Giemsa staining until they reached a parasitaemia of >1%. Values show the mean of three biological replicates (+/- SD).

2.4.4 Lack of S/M-phase entry correlates with decelerated transcriptome progression in G1-phase

We next conducted genome-wide transcriptional profiling of paired synchronous 3D7/HP1^{ON} and 3D7/HP1^{OFF} cultures at eleven consecutive time points (TPs) spanning generations 1 and 2 to study (i) the effect of PfHP1 on heritable gene silencing; and (ii) to identify the PfHP1-dependent pathway responsible for gametocyte conversion (Figure 4A and Table S1). Until 16-24hpi in generation 2, the corresponding transcriptomes were highly comparable between both populations (Figure 4A) and progressed with similar kinetics through the first IDC and second generation ring stage development (TPs 2-9) (Figures 4B and 4C). In

contrast, at 24-48 hours after re-invasion (TPs 10-12), when 3D7/HP1^{ON} parasites went through schizogony and the 3D7/HP1^{OFF} population consisted of a mixture of early gametocytes and arrested trophozoites, the transcriptomes correlated poorly (Figure 4A) and parasites failed to launch a schizont-specific transcription profile (Figures 4B and 4C). This slowdown in transcriptome development reflects a substantial deceleration in G1 progression and failure to enter S-phase in generation 2, which is consistent with the growth phenotype observed for 3D7/HP1^{OFF} parasites.

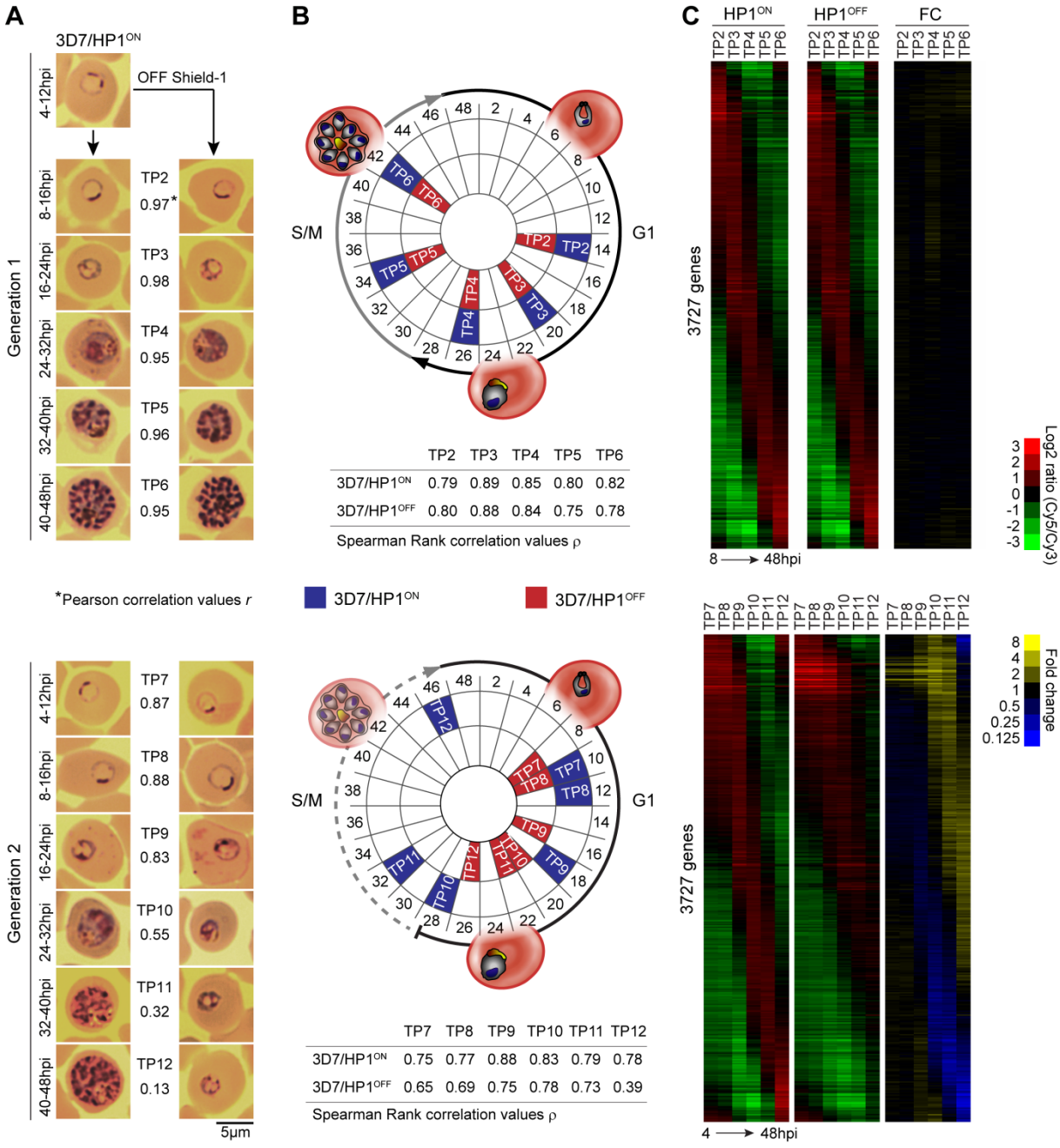

Figure 4. PfHP1-depletion leads to a marked slowdown in transcriptome progression

(A) 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites were sampled for comparative transcriptome analysis. Pairwise correlation between transcriptomes of corresponding TPs is indicated by Pearson correlation coefficients *r* (asterisk). (B) Mapping of experimental transcriptomes to a high-resolution reference

dataset (Mok et al., 2011). Blue and red boxes identify the best-fit TP (hpi) in a high-resolution reference dataset for each 3D7/HP1^{ON} and 3D7/HP1^{OFF} transcriptome, respectively. Spearman rank coefficients (ρ) are provided. See also Supplemental Experimental Procedures. (C) Comparison of global temporal expression profiles in generation 1 and 2. Heat maps are ordered according to the phase calculated for 3D7/HP1^{ON} parasites (TPs 7-12, starting at -π/2) and display relative gene expression levels (red/green) and fold changes (FC) in gene expression (yellow/blue). See also Table S1 and Supplemental Experimental Procedures.

2.4.5 PfHP1 silences heterochromatic genes and is essential for the maintenance of singular *var* gene choice

To identify genes differentially expressed in direct response to PfHP1 depletion we focused our analysis on the comparable growth phase ranging from generation 1 trophozoites to late ring stages in generation 2 (TPs 4-9). Consistent with the conserved role for HP1 in heritable gene silencing, we observed a general de-repression of heterochromatic genes in 3D7/HP1^{OFF} parasites and 113 PfHP1-associated genes (31.2%) displayed a significant increase in mean expression (>1.5-fold, fdr<0.1) compared to 3D7/HP1^{ON} parasites (Figure 5A and Table S1). In contrast, only 16 euchromatic genes (0.34%) were differentially expressed, of which four up-regulated genes had previously been associated with early gametocyte development; PF3D7_1102500 (*phistb*; GEXP02), PF3D7_1335000 (*msrp1*), PF3D7_1472200 (class II histone deacetylase (HDAC)), and PF3D7_1473700 (*nup116*) (Silvestrini et al., 2010; Eksi et al., 2012) (Figure 5B and Table S1). Strikingly, the strongest de-repression was observed for the *var* gene family; 52 out of 60 members were significantly and highly up-regulated in PfHP1-depleted parasites. In addition, many *rifin* and *pfmc-2tm* genes and several members of other subtelomeric gene families were significantly induced, and even among the non-significantly de-regulated heterochromatic genes the majority was still up-regulated in absence of PfHP1 (Figures 5A and S3).

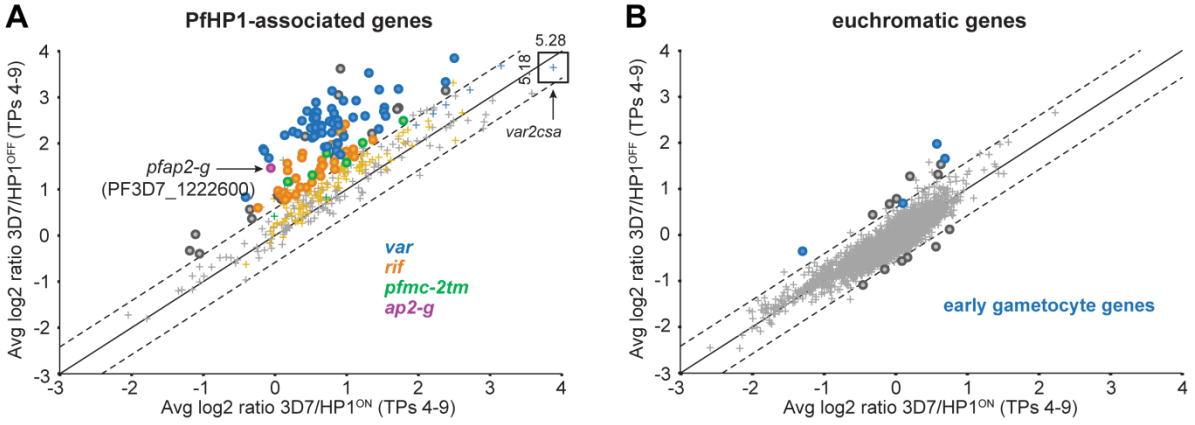

Figure 5. PfHP1 depletion leads to de-repression of PfHP1-associated genes

(A) Scatter plot comparing mean relative expression values of all 362 PfHP1-associated genes. Significantly de-regulated genes are indicated by circles (>1.5-fold; fdr<0.1). See also Figure S3 and

Table S1. (B) Scatter plot comparing mean relative expression values of all 4771 euchromatic genes. Significantly de-regulated genes are indicated by circles (>1.5-fold; fdr<0.1). See also Table S1.

We next investigated the prevailing role of PfHP1 in *var* gene silencing in more detail. Removal of Shield-1 at 4-12hpi had no immediate effect on *var* transcription in generation 1 (Figure 6A), which is explained by the persistent binding of PfHP1 to chromatin shortly after Shield-1 withdrawal (Figure 1D). *var* transcription was also unchanged in schizonts demonstrating that *var* promoters retain their ring stage-specific activation profile even in absence of PfHP1. By contrast, almost all *var* genes were massively up-regulated after re-invasion and individual genes showed up to 30-fold higher expression levels (Figure 6A). Importantly, however, the few *var* genes already dominantly expressed in 3D7/HP1^{ON} parasites, most notably *var2csa* (PF3D7_1200600) (Salanti et al., 2003), were not or only slightly induced (Figures 6A and S4). This proves that *var* activation was not due to transcriptional switches but that all *var* genes were active simultaneously in 3D7/HP1^{OFF} parasites. Consistent with these findings PfHP1-depleted parasites co-expressed several PfEMP1 variants of different sizes, whereas 3D7/HP1^{ON} parasites predominantly expressed a single protein consistent with the size of VAR2CSA (Figure 6B). IFAs further corroborated hyper-expression of PfEMP1 in 3D7/HP1^{OFF} parasites at the single cell level and indicated correct trafficking of PfEMP1 to the iRBC surface (Figure 6C).

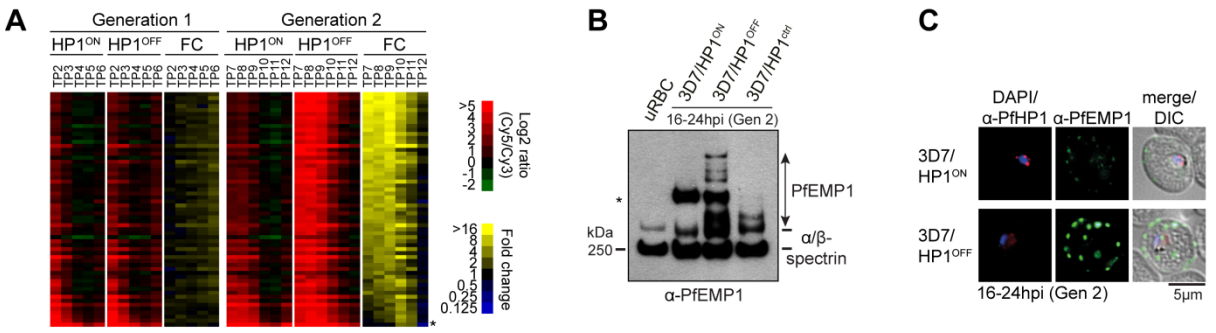

Figure 6. PfHP1 is required for heritable *var* gene silencing and maintenance of singular *var* gene choice

(A) Temporal progression of relative abundance (red/green) and fold change (FC) in expression (yellow/blue) for all *var* genes in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites across all eleven TPs analysed. Asterisk, *var2csa*. See also Figure S4 and Table S1. (B) PfEMP1 expression in 3D7/HP1^{ON}, 3D7/HP1^{OFF} and 3D7/HP1^{ctrl} parasites at 16-24hpi in generation 2. Equal cell numbers were analysed in each lane. The pan-specific α -PfEMP1 antibody (mAb 6H1) was raised against a part of the C-terminal ATS domain that is conserved among PfEMP1 variants (Duffy et al., 2002). uRBC, uninfected RBCs (note that α-PfEMP1 antibodies cross-react with human spectrin). (C) α-PfHP1/α-PfEMP1 (mAb 6H1) IFAs of 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites at 16-24hpi in generation 2.

2.4.6 Sexual differentiation is linked to the PfHP1-dependent de-repression of an ApiAP2 transcription factor

Interestingly, a single member of the *apiap2* family of genes encoding phylum-specific transcription factors (TFs) (Balaji et al., 2005) was also significantly de-repressed in 3D7/HP1^{OFF} parasites (Figure 5A). This *apiap2* gene (PF3D7_1222600) represents the only PfHP1-associated member of the family (Flueck et al., 2009) and encodes the TF AP2-G that is essential for gametocyte conversion in *P. falciparum* and *P. berghei* (Kafsack et al., 2014; Sinha et al., 2014). Moreover, among all de-regulated PfHP1-associated loci, *pfap2-g* was the only gene that does not encode a surface antigen or exported protein (Table S1). We observed that *pfap2-g* de-repression was already initiated in 3D7/HP1^{OFF} generation 1 schizonts (32-40hpi) coincident with the dissociation of PfHP1 from the *pfap2-g* locus (Figure 7A). Importantly, when 3D7/HP1^{OFF} parasites were allowed to re-accumulate PfHP1 prior to schizogony (28-36hpi) gametocyte hyper-conversion was prevented (Figures 7B and S5). Restoring PfHP1 expression at 34-42hpi was only moderately effective in preventing sexual commitment, and parasites rescued at 40-48hpi or after re-invasion showed a hyper-conversion phenotype similar to non-rescued parasites. The temporal correlation between de-repression of *pfap2-g* and gametocyte commitment during schizogony, together with the fact that both processes are strictly PfHP1-dependent, identify the targeted activation of PfAP2-G as the key mechanism responsible for sexual conversion.

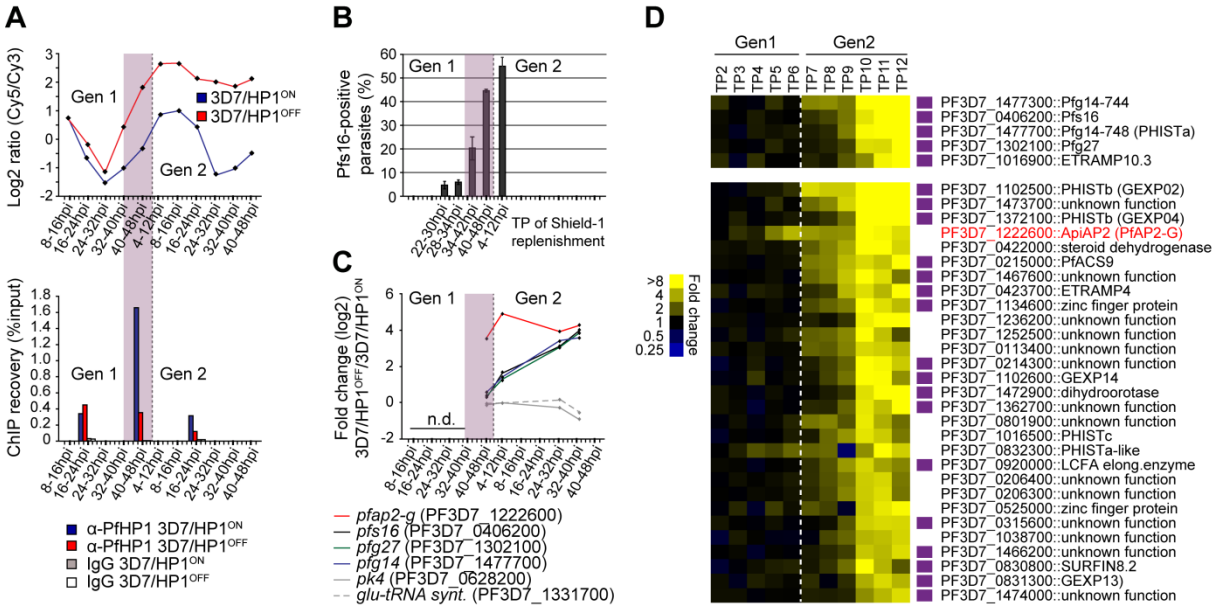

Figure 7. Gametocyte differentiation is linked to the PfHP1-dependent activation of *pfap2-g*

(A) Temporal expression profile (Cy5/Cy3 log2 ratios) of *pfap2-g* (top) and ChIP-qPCR results showing PfHP1 occupancy at the *pfap2-g* locus (bottom). The sexual commitment phase is highlighted in purple. See also Figure S6 and Table S1. (B) Proportion of Pfs16-/KAHRP-positive parasites in 3D7/HP1^{OFF} populations rescued at different TPs in generation 1 (x-axis) as determined by IFA at 32-40 hours post re-invasion in generation 2. Values show the mean (+/-SD) of three biological replicates.

See also Figure S5. (C) Induction of *pfap2-g* and the three early gametocyte markers *pfs16* (PF3D7_0406200) (Bruce et al., 1994), *pfg27* (PF3D7_1302100) (Alano et al., 1991), and *pfg14_748* (PF3D7_1477700) (Eksi et al., 2005) in 3D7/HP1^{OFF} compared to 3D7/HP1^{ON} parasites as determined by qRT-PCR on biological replicate samples. Negative control genes are in grey. n.d., not determined. (D) Temporal progression of fold changes in expression of known (upper heat map) and newly predicted (lower heat map) early gametocyte genes in 3D7/HP1^{OFF} compared to 3D7/HP1^{ON} parasites. Early gametocyte genes identified in previous studies are highlighted in purple. See also Figure S7, Table S1 and Supplemental Experimental Procedures.

2.4.7 PfHP1 depletion results in reduced H3K9me3 levels at heterochromatic loci

HP1-dependent recruitment of SU(VAR)3-9-type HKMTs is essential for the spreading and inheritance of H3K9me3 marks in model eukaryotes (Grewal and Jia, 2007). We therefore tested if the local depletion of PfHP1 caused a reduction of H3K9me3 levels. Indeed, ChIP-qPCR experiments demonstrated that H3K9me3 occupancy was substantially reduced at *var* genes and the *pfap2-g* locus in 3D7/HP1^{OFF} parasites, both in generation 1 schizonts and generation 2 ring stages (Figure S6). Noteworthy, the drop in H3K9me3 enrichment at individual loci was equally pronounced as the depletion of PfHP1 itself. As expected, PfHP1 and H3K9me3 were not associated with the early gametocyte marker *pfs16* in both cultures at both TPs, which confirms that upregulation of *pfs16* in early gametocytes is PfHP1-independent and rather occurs as a result of a gametocyte-specific transcriptional programme. Together, these findings show that, in analogy to other eukaryotes, PfHP1 is required for the local deposition and inheritance of H3K9me3 marks on newly replicated chromatin. This is likely mediated by the PfHP1-dependent recruitment of a H3K9-specific HKMT (probably PfSET3 (Volz et al., 2010; Lopez-Rubio et al., 2009)). However, confirmation of PfSET3 as functional SU(VAR)3-9 homolog as well as a possible physical interaction of this factor with PfHP1 remains to be determined.

2.4.8 Identification of genes associated with early gametocyte development

Our experimental setup combined with the high rate of synchronous gametocyte induction in PfHP1-depleted parasites allowed us for the first time to identify transcriptional events linked to gametocyte conversion in real time and based on comparison of two isogenic clones. Following de-repression of *pfap2-g* in generation 1, known markers of early sexual development were up-regulated only after re-invasion (Table S1) and this was confirmed by qRT-PCR (Figure 7C). We therefore queried our dataset to identify genes induced upon gametocyte conversion and identified 29 additional early gametocyte candidate genes (Figure 7D and Table S1). Notably, 17 (58.6%) of these genes have been linked to early sexual development in previous high-throughput studies (Silvestrini et al., 2005; Silvestrini et al., 2010; Eksi et al., 2012; Young et al., 2005), which underpins the high accuracy and

stringency of our search. qRT-PCR confirmed up-regulation of these genes in 3D7/HP1^{OFF} parasites and showed that apart from *pfap2-g* only one additional gene (PF3D7_0832300; *phista*-like) was up-regulated already during the commitment phase. In contrast, induction of all other genes was delayed until the sexual ring stage and increased further during stage I gametocyte development (24-40hpi) (Figures 7D and S7). Finally, we tested if these candidate genes are also up-regulated in naturally induced gametocytes by comparing their transcription between 3D7 wild type parasites and a gametocyte-deficient clone of 3D7 (F12) (Alano et al., 1995). Indeed, all predicted genes showed consistently higher transcription levels in 3D7 compared to F12, which ultimately confirms that their activation is related to early gametocyte differentiation (Figure S7).

2.5 Discussion

Our study shows that PfHP1 is strictly required to propagate non-permissive heterochromatin to daughter cells in order to silence a vast antigenic repertoire, and in particular to perpetuate mutually exclusive *var* transcription. Since the landmark discovery of the *var* gene family (Su et al., 1995; Baruch et al., 1995; Smith et al., 1995) a large number of studies firmly established that antigenic variation in *P. falciparum* is controlled by a complex epigenetic strategy involving reversible histone modifications, chromatin remodeling and locus re-positioning (Lopez-Rubio et al., 2007; Jiang et al., 2013; Freitas-Junior et al., 2005; Tonkin et al., 2009; Duraisingh et al., 2005; Petter et al., 2011; Voss et al., 2006; Ralph et al., 2005; Lopez-Rubio et al., 2009). Together, these findings support a model in which singular *var* gene choice is achieved by restricting transcription of a single locus to an elusive perinuclear *var* expression site (VES), and where switching occurs through competitive replacement of the active gene with a previously silenced member. How these different processes and layers of regulation are interconnected to control antigenic variation, however, is only poorly understood.

Here, we demonstrate that depletion of PfHP1 during schizogony leads to the simultaneous activation of all *var* genes and concomitant hyper-expression of PfEMP1 in daughter parasites. This shows that PfHP1 is required to protect *var* genes from activation outside the VES, which is further supported by the fact that *var* promoter fragments activate stage-specific transcription by default when placed upstream of the transcriptional start site of a euchromatic gene (Brancucci et al., 2012). Hence, unlike in African trypanosomes (Navarro and Gull, 2001), the functional principle of the VES is not based on the sequestration of exclusive transcription machinery but rather depends on histone modifying and remodelling activities capable of disassembling heterochromatin at a single locus. This concept is consistent with the recent description of the H3K4me-specific methyltransferase (HKMT) PfSET10 that localises exclusively to the VES (Volz et al., 2012). Note that mutually

exclusive *var* transcription is also disrupted in parasites lacking expression of the H3K36-specific HKMT PfSET2 (also known as PfSETvs) (Jiang et al., 2013). Interestingly, Jiang and colleagues observed a reduction in H3K36me3 as well as H3K9me3 occupancy at active *var* loci in ΔPfSET2 parasites, suggesting functional interdependence of different epigenetic control processes in regulating antigenic variation. Taken together, these results fill an important gap in our understanding of the regulatory mechanisms underlying mutually exclusive *var* gene transcription and antigenic variation of PfEMP1 and will be instrumental for the further functional dissection of this important immune evasion strategy. Moreover, the PfEMP1 hyper-expression phenotype reported here will serve as a useful tool to study PfEMP1-based pathogenesis and immunity and may provide new opportunities for the development of a malaria vaccine. Noteworthy, in an analogous system, immunisation with mutant *Giardia lamblia* parasites co-expressing many variant-specific surface proteins has successfully been applied to induce strain-transcendent protective immunity in an experimental infection model (Rivero et al., 2010).

In addition to virulence gene silencing, we also identified an essential role for PfHP1 in mitotic proliferation. In absence of PfHP1, asexual trophozoites fail to proliferate and enter a state of cell cycle arrest that is reversible in a PfHP1-dependent manner. Although the exact pathway in which PfHP1 is required for cell cycle progression remains unknown, the lack of significant levels of DNA synthesis in PfHP1-depleted trophozoites is indicative of defects in S-phase entry or progression. Indeed, HP1 directly interacts with several factors involved in pre-replicative complex assembly and replication initiation or elongation (e.g. CDC18/CDC6, ORCs, MCMs, CAF1) in model eukaryotes (Kwon and Workman, 2008; Christensen and Tye, 2003; Li et al., 2011). Moreover, loss of HP1 function causes delayed replication timing and/or S-phase progression defects in *S. pombe*, *D. melanogaster* and mouse cells (Hayashi et al., 2009; Schwaiger et al., 2010; Quivy et al., 2008). Hence, it is conceivable that PfHP1 may be essential for DNA replication in *P. falciparum* and further experiments are now required to test this intriguing hypothesis in more detail.

Remarkably, we demonstrate that PfHP1's capacity to regulate the bistable transcription of a single euchromatic gene balances mitotic proliferation and sexual differentiation of malaria blood stage parasites. In this context it is noteworthy that silencing of heterochromatic genes in *P. falciparum* is functionally dependent on the sirtuin HDACs PfSIR2A/PfSIR2B (Tonkin et al., 2009; Duraisingh et al., 2005), PfSET2 (Jiang et al., 2013) and the class II HDAC PfHDA2 (Coleman and Skillman et al., this issue). Of these histone-modifying enzymes, however, only PfHDA2 also controls *pfap2-g* expression suggesting that PfHP1 and PfHDA2 cooperate in a distinct silencing pathway to also regulate euchromatic genes. It will therefore be interesting to test if PfHP1 and PfHDA2 occur together in a specific silencing complex. Indeed, several class II HDACs interact directly with HP1 and are important for HP1-

dependent gene silencing in model eukaryotes (Yamada et al., 2005; Zhang et al., 2002). We propose that epigenetic silencing of *pfap2-g* promotes continuous mitotic proliferation and antagonises sexual conversion, while local dissociation of PfHP1 from the *pfap2-g* locus activates PfAP2-G expression and triggers sexual conversion and gametocyte differentiation. In analogy to the essential role of ApiAP2 TFs in stage-specific gene expression and parasite development in other life cycle stages (Yuda et al., 2009; Iwanaga et al., 2012; Yuda et al., 2010), PfAP2-G likely regulates a transcriptional response effecting gametocyte development and cell cycle exit. In both *P. falciparum* and *P. berghei*, PfAP2-G binding motifs were indeed found enriched in the upstream region of genes associated with sexual differentiation, and the occurrence of the respective target sites upstream of *pfap2-g* itself further indicates that PfAP2-G may establish an autoregulatory feedback loop (Kafsack et al., 2014; Sinha et al., 2014). Interestingly, we demonstrate that transcriptional changes associated with the early phase of differentiation are limited to a small number of genes, but become more pronounced once gametocytes enter stage I development. We explain this by the fact that both asexual and sexually committed schizonts need to produce invasive merozoites capable of establishing RBC infection. In fact, many of the early gametocyte genes predicted here and elsewhere (Eksi et al., 2005; Silvestrini et al., 2010) code for proteins implicated in host cell remodeling, which is indicative for the requirement of gametocyte-specific host cell modifications. While it is possible that PfAP2-G regulates some/all of these genes directly, genome-wide ChIP approaches will be necessary for a comprehensive identification of PfAP2-G target genes and understanding of PfAP2-G function.

Our results reveal important novel mechanistic insight into the pathway underlying sexual commitment and identify a new concept of HP1-dependent cell differentiation in unicellular eukaryotes. Interestingly, the blood stage of infection is the only phase of the entire life cycle where parasites have a choice to enter either one of two developmental pathways. It thus appears likely that the epigenetic basis for this switch evolved to adapt sexual conversion rates for optimal transmission during the course of infection. Indeed, gametocyte conversion rates are highly variable between different isolates and clones and influenced by a broad spectrum of environmental conditions (Baker, 2010; Alano and Carter, 1990). Although the molecular factor(s) triggering gametocyte conversion have not been identified, two recent studies reported that cell-cell communication via exosomes/microvesicles causes a dramatic increase in gametocyte conversion (Regev-Rudzki et al., 2013; Mantel et al., 2013). It is therefore tempting to speculate that cargo delivered by these vesicles may trigger a signaling cascade that ultimately evicts PfHP1 from the *pfap2-g* locus.

In conclusion, we identified PfHP1 as an essential factor in mitotic proliferation and as a key mediator of two systems of CVGE that secure the survival and transmission of malaria blood stage parasites, respectively. Our data provide important mechanistic insight into the

regulatory processes underlying antigenic variation and sexual conversion and generate knowledge relevant for investigating conceptually similar systems in other eukaryotes. Importantly, we established for the first time that gametocyte commitment is epigenetically regulated. This significant discovery will facilitate the targeted dissection of the molecular pathway triggering sexual conversion and has major implications for the identification of novel approaches to prevent malaria transmission.

2.6 Experimental Procedures

2.6.1 Parasite culture and transfection

P. falciparum 3D7 cell culture and transfection was performed as described (Trager and Jensen, 1978; Lambros and Vanderberg, 1979; Voss et al., 2006). Transfection constructs are described in the Supplemental Experimental Procedures section and in Figure S1. Parasites were grown in presence of indicated combinations of 4nM WR99210 (WR) and 625nM Shield-1. 3D7/HP1^{ON} clones were obtained by limiting dilution.

2.6.2 Western Blot analysis

Nuclei were isolated as described (Voss et al., 2003) and lysed in 2% SDS, 10mM Tris, 1mM DTT (pH 8.0). Proteins were detected using rabbit α-PfHP1 1:5,000 (Figure S1 and Supplemental Experimental Procedures) and α-H4 (Abcam ab10158), 1:10,000. PfEMP1 was extracted as described (van Schravendijk et al., 1993) and detected using the pan-specific α-PfEMP1 mouse mAb 1B/6H-1 (1:500) (Duffy et al., 2002).

2.6.3 Fluorescence Microscopy

Live cell fluorescence microscopy and IFAs were performed as described (Witmer et al., 2012). IFAs were performed on methanol-fixed cells using mouse IgG2a mAb α-HRP1 (α-KAHRP) (kind gift from D. Taylor), 1:500; mouse IgG1 mAb α-Pfs16 (kind gift from Robert W. Sauerwein), 1:250; mouse IgG1 mAb α-PfEMP1 (1B/6H-1) (Duffy et al., 2002), 1:150; rabbit α-PfHP1, 1:100. Secondary antibody dilutions were: Alexa-Fluor® 568-conjugated α-rabbit IgG (Molecular Probes) 1:250; Alexa-Fluor® 568-conjugated α-mouse IgG2a (Molecular Probes) 1:250; Alexa-Fluor® 488-conjugated α-mouse IgG1 (Molecular Probes) 1:250; FITC-conjugated α-mouse IgG (Kirkegaard Perry Laboratories) 1:250. Images were taken at 96-fold magnification on a Leica DM 5000B microscope with a Leica DFC 300 FX camera and acquired via the Leica IM 1000 software and processed using Adobe Photoshop CS6. For each experiment, images were acquired and processed with identical settings.

2.6.4 Isothermal Microcalorimetry

Isothermal microcalorimetry measures the heat flow produced by a biological sample over time. Isothermal microcalorimetry experiments and data analysis were performed as described using a Thermal Activity Monitor (Model 3102 TAM III, TA Instruments, New Castle, DE, USA) with minor modifications (Wenzler et al., 2012) (see Supplemental Experimental Procedures).

2.6.5 Quantitative Reverse Transcription PCR (qRT-PCR)

3D7/HP1^{ON} parasites were synchronised twice 16 hours apart to obtain an eight hour growth window and then split into two populations at 4-12hpi and cultured either in presence or absence of Shield-1. 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites were harvested at 40-48hpi in generation 1 and at three consecutive time points in generation 2 (4-12hpi, 24-32hpi, 32-40hpi). 3D7 and F12 populations were synchronised identically and time points were harvested at 4-12hpi, 24-32hpi and 40-48hpi. Isolation and processing of total RNA and qRT-PCR was conducted as described with minor modifications (Witmer et al., 2012) (see Supplemental Experimental Procedures).

2.6.6 Targeted Chromatin Immunoprecipitation (ChIP-qPCR)

3D7/HP1^{ON} parasites were synchronised twice 16 hours apart to obtain an eight hour growth window and then split into two populations, one of which was taken off Shield-1 at 4-12hpi. Sample pairs were harvested at 16-24hpi and 40-48hpi in generation 1, and at 16-24hpi in generation 2. Isolation of formaldehyde-crosslinked chromatin and ChIP-qPCR analysis was performed as described with minor modifications (Flueck et al., 2009) using 0.6µg affinity-purified α-PfHP1, 5µg α-H3K9me3 (Millipore 07_442), or 5µg rabbit IgG negative control antibodies (Millipore 12–370) (see Supplemental Experimental Procedures).

2.6.7 Flow Cytometry

Tightly synchronised 3D7/HP1^{ON} parasites were split at 0-4hpi and cultured either in presence or absence of Shield-1. At 20-24hpi after re-invasion the 3D7/HP1^{ON} and 3D7/HP1^{OFF} populations were synchronised again to obtain a four hour growth window. DNA content analysis was carried out on five consecutive TPs in generation 2, starting at 24-28hpi. 100µl packed RBCs were fixed in 4% formaldehyde/0.015% glutaraldehyde, washed three times in RPMI-Hepes, incubated in 1ml RPMI-Hepes, 0.1% Triton X-100, 0.1µg/ml RNAse A, 20µM FxCycle^{TM} Far Red stain (Molecular Probes) for 30min in the dark, and analysed using an AccuriC6 instrument (BD Biosciences). A minimum of 4’000 gated iRBCs

were measured (excitation 640nm; 30mW diode; emission detection FL4 675nm +/-12.5nm). Acquired data were processed using FlowJo software (Version 10.0.5).

2.6.8 Microarray experiments and data analysis

RNA extraction and cDNA synthesis was carried out as described (Bozdech et al., 2003). Cy5-labelled test cDNAs were hybridised against a Cy3-labelled 3D7 cDNA reference pool that was made by combining total RNA isolated from five consecutive time points across the IDC. Equal amounts of Cy5- and Cy3-labelled samples were hybridised on a *P. falciparum* glass slide microarray containing 10416 70-mer ORF probes (Hu et al., 2007). Hybridisation was carried out at 65°C in an automated hybridisation station (Maui, USA) for at least 12 hours (Bozdech et al., 2003). Slides were washed twice in 0.5xSSC/0.02% SDS, once in 0.05xSSC, spun dry and scanned using the GenePix scanner 4000B and GenePix pro 6.0 software (Axon Laboratory). Detailed protocols describing microarray re-annotation, data processing and analysis are provided in the Supplemental Experimental Procedures section. The microarray data reported in this paper are accessible through the Gene Expression Omnibus Series accession number GSE53176 at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53176

2.7 Supplementary Information

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found in paragraph 2.7 or with this article online at: http://dx.doi.org/10.1016/j.chom.2014.07.004. Please note that Table S1 is not included into the thesis due to size and can only be found online.

2.8 Author Contributions

N.M.B.B. and N.L.B. designed and performed experiments, analysed data, prepared illustrations and wrote the paper. W.H.C. performed microarray hybridizations. I.N. expressed recombinant PfHP1 and produced affinity-purified α-PfHP1 antibodies. L.Z. analysed microarray data. R.W. designed and performed qRT-PCR experiments. C.F. performed isothermal microcalorimetry experiments. M.R., I.F. and Z.B. designed experiments and interpreted data. T.S.V. supervised the study, designed experiments, analysed data, prepared illustrations and wrote the manuscript.

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2.11 Supplemental Information

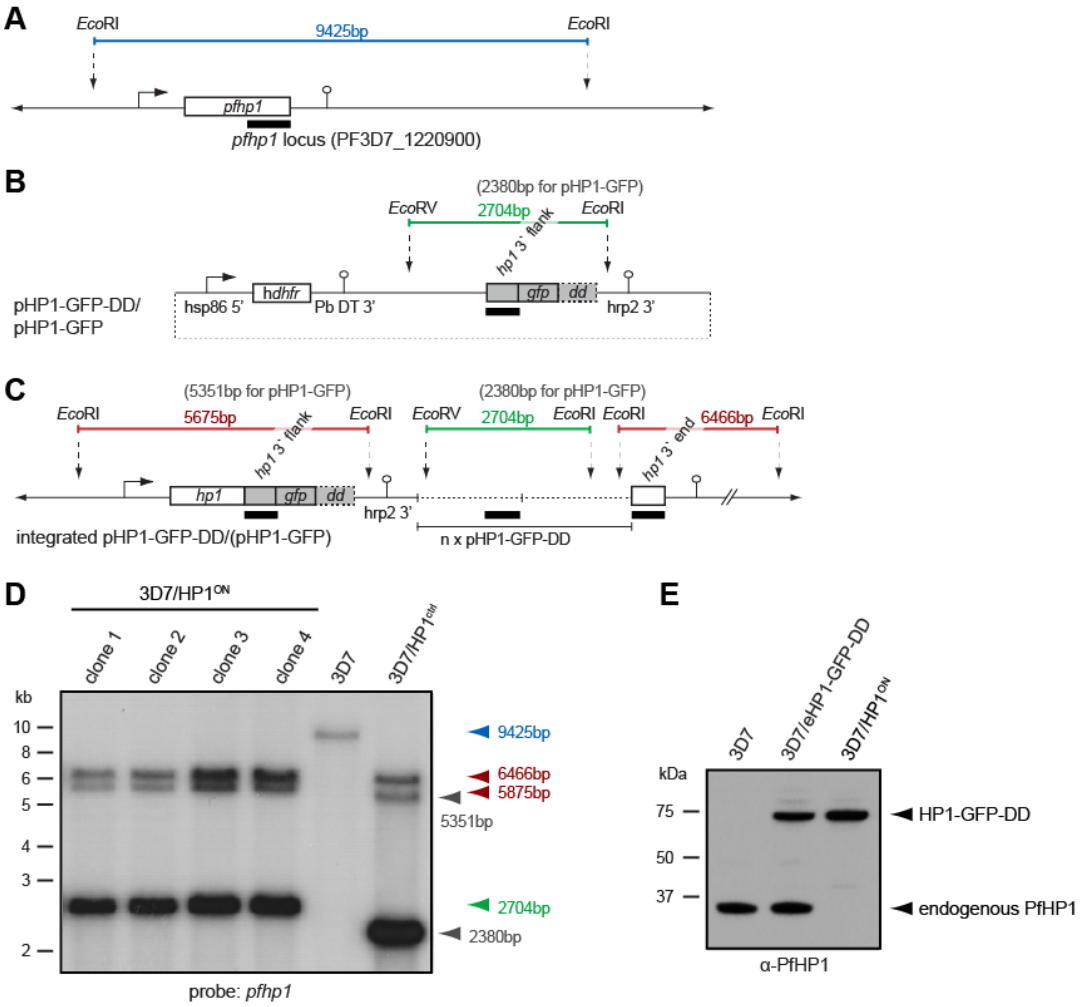

Figure S1. Plasmid maps and Southern blot analysis (related to Figure 1)

(A) Schematic map of the endogenous *pfhp1* locus (PF3D7_1220900). (B) Schematic map of the pHP1-GFP-DD and pHP1-GFP (control) plasmid. (C) Schematic map of the endogenous *pfhp1* locus (PF3D7_1220900) after plasmid integration in 3D7/HP1^{ON} and 3D7/HP1^{ctrl} parasites. *EcoR*I and *EcoR*V restriction sites and fragment lengths are indicated and colour-coded. The hybridisation probe is shown by a black solid line. Dashed lines indicate integration of multiple tandem plasmid copies. hsp86 5', heat shock protein 86 gene promoter; h*dhfr*, human dihydrofolate reductase; Pb DT 3', *P. berghei dhfr*-thymidilate synthase gene terminator; *gfp*, green fluorescent protein; *dd*, destabilisation domain; hrp2 3', histidine-rich protein 2 gene terminator. (D) Southern blot showing plasmid integration into the endogenous *pfhp1* locus in four 3D7/HP1^{ON} clones and the 3D7/HP1^{ctrl} population. 3D7 wild-type gDNA was used as a control. gDNA was digested with *EcoR*I and *EcoR*V, transferred to a Nylon membrane (GE Healthcare) and hybridised with a ^{32}P-dATP-labelled *pfhp1* fragment. (E) Specificity of the polyclonal affinity-purified anti-PfHP1 antibody. α-PfHP1 Western blot of nuclear proteins extracted from 3D7 wild-type parasites (lane 1), from parasites expressing episomal PfHP1-GFP-DD in addition to endogenous PfHP1 (3D7/eHP1-GFP-DD^{ON}) (unpublished) (lane 2), and from 3D7/HP1^{ON} parasites expressing PfHP1-GFP-DD from the endogenous locus (lane 3). See also Supplemental Experimental Procedures.

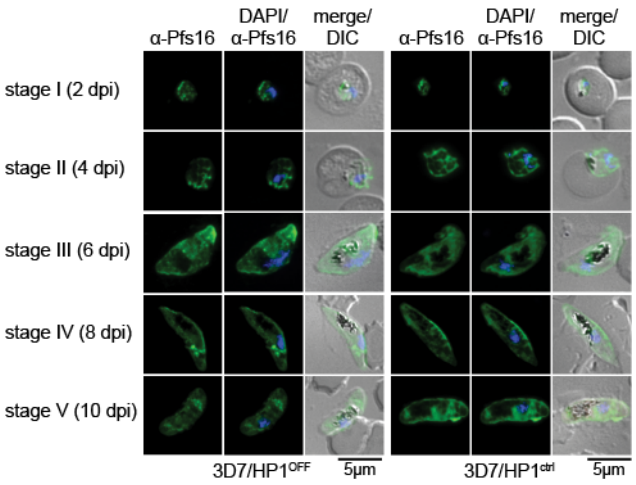

Figure S2. Development of PfHP1-depleted gametocytes (related to Figure 2)

Morphology and development of 3D7/HP1^{OFF} and 3D7/HP1^{ctrl} stage I-V gametocytes as assessed by IFA using α-Pfs16 antibodies. dpi, days post re-invasion.

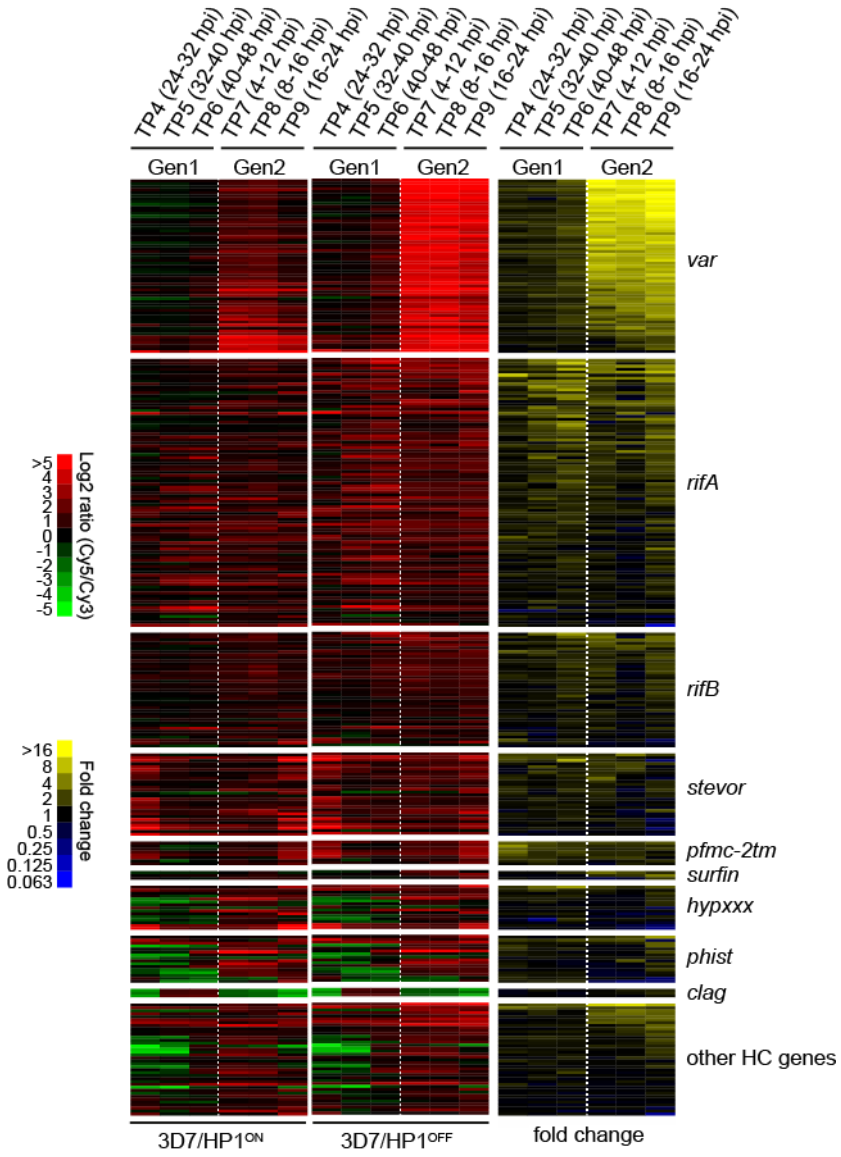

Figure S3. PfHP1-depletion causes de-repression of heterochromatic genes (related to Figure 5A)

Temporal relative expression profiles (red/green) and fold change in gene expression (yellow/blue) of all heterochromatic genes in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites in generation 1 and 2 (TPs 4-9). Heat maps are grouped into subtelomeric gene families and ordered according to decreasing mean fold induction in 3D7/HP1^{OFF} parasites. See also Table S1.

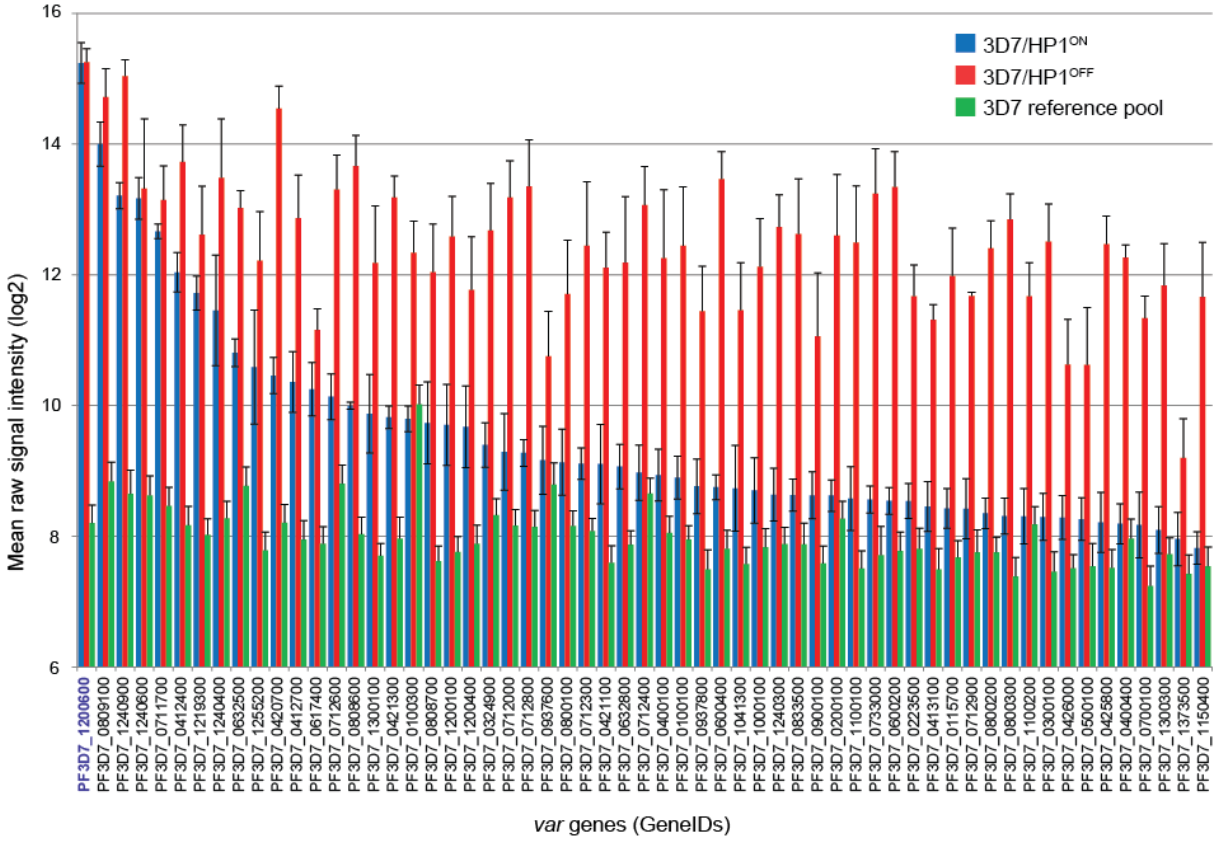

Figure S4. Absolute *var* transcript abundance in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites (related to Figure 6A)

Estimation of absolute *var* transcript abundance in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites. Values represent the mean raw signal intensities for all *var* genes for TPs 7-9 in 3D7/HP1^{ON} and 3D7/HP1^{OFF} ring stage parasites (Cy5), and for all eleven 3D7 reference pool hybridisations (Cy3) (+/- SD). Genes are ordered according to decreasing signal intensity in 3D7/HP1^{ON} parasites. The most dominant *var* transcript in 3D7/HP1^{ON} parasites (*var2csa*) is highlighted in blue.

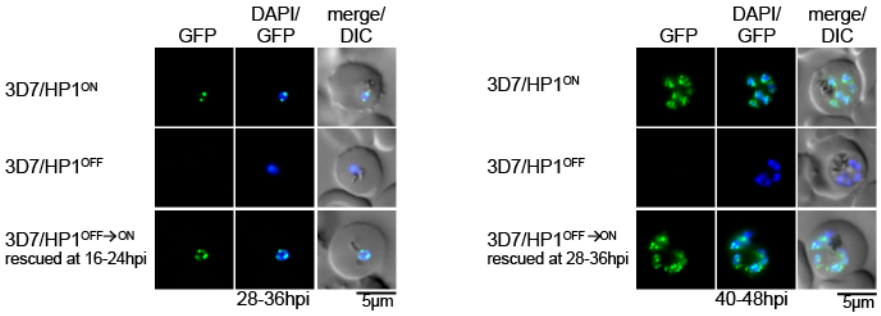

Figure S5. PfHP1 expression is rapidly restored upon Shield-1 replenishment (related to Figure 7B)

Live fluorescence microscopy demonstrates the rapid re-accumulation of PfHP1-GFP-DD in rescued 3D7/HP1$^{OFF\rightarrow ON}$ parasites. PfHP1 depletion was induced 4-12hpi by Shield-1 removal (3D7/HP1^{OFF}) and subsequently restored (3D7/HP1$^{OFF\rightarrow ON}$) by replenishing Shield-1 at 16-24hpi and 28-36hpi, respectively.

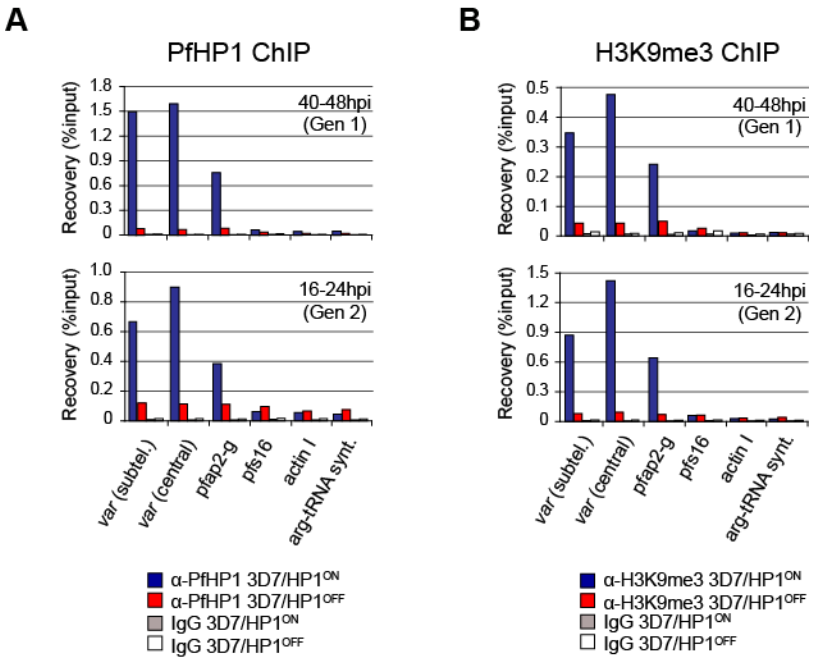

Figure S6. PfHP1 depletion causes substantial reduction in H3K9me3 marks at heterochromatic loci (related to Figures 1D and 7A)

ChIP-qPCR results showing PfHP1 (A) and H3K9me3 (B) occupancy at selected loci in 3D7/HP1^{ON} and 3D7/HP1^{OFF} (Shield-1 removal at 4-12hpi) parasites in generation 1 schizonts (40-48hpi; upper panels) and generation 2 ring stages (16-24hpi; lower panels). Tested loci include subtelomeric and chromosome-central *var* genes, the gametocyte-specific genes *pfap2-g* and *pfs16* as well two euchromatic control loci. Note that in contrast to *pfap2-g*, the *pfs16* locus is naturally devoid of PfHP1/H3K9me3 marks. Values represent the proportion of chromatin recovered from the input samples. Rabbit IgG antibodies were used as a negative control. These data were obtained from biological replicates of the samples tested in Figure 1D and 7A.

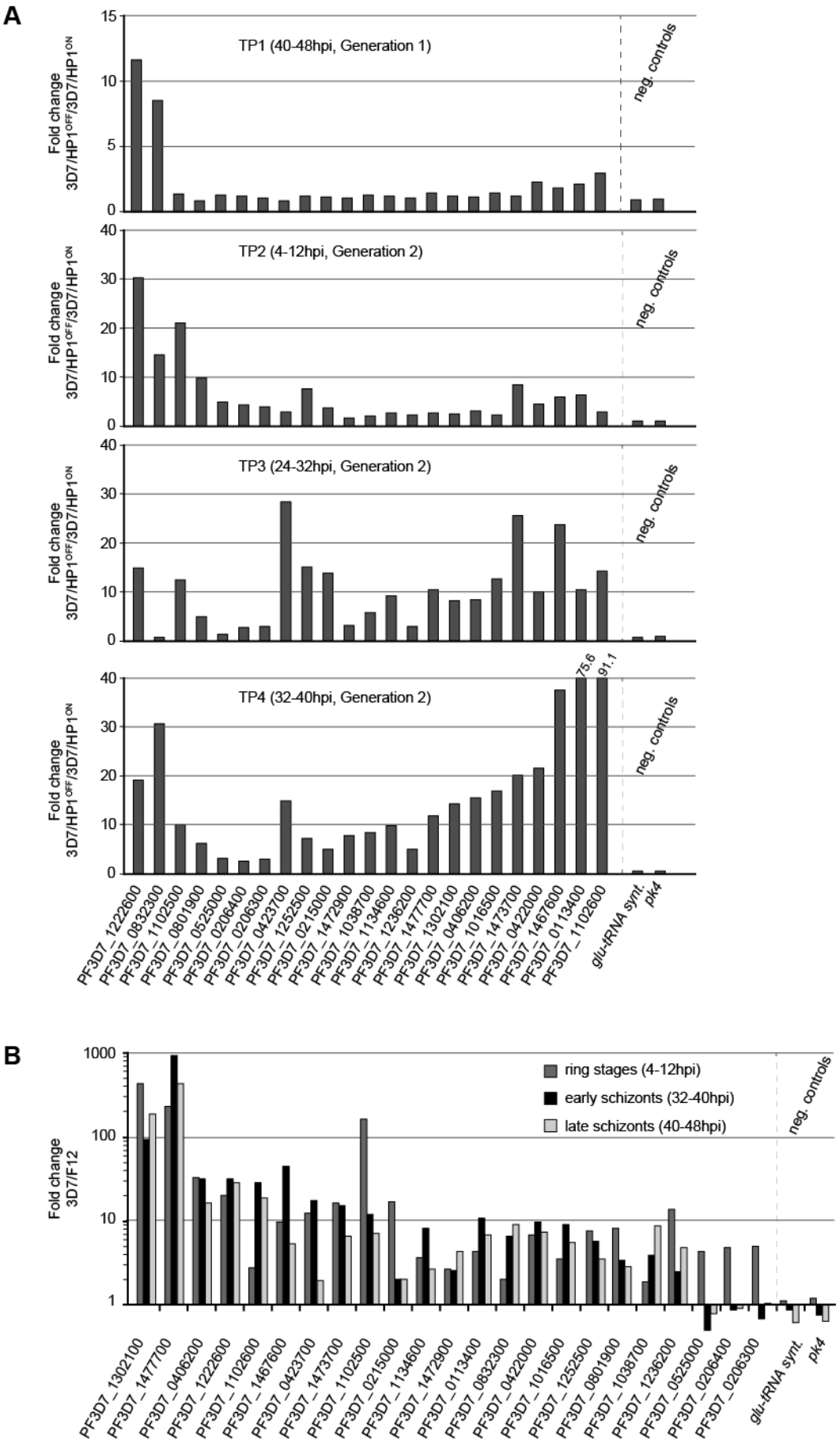
A
TP1 (40-48hpi, Generation 1)
TP2 (4-12hpi, Generation 2)
TP3 (24-32hpi, Generation 2)
TP4 (32-40hpi, Generation 2)
Fold change 3D7/HP1OFF/3D7/HP1ON
neg. controls
75.6
91.1
PF3D7_1222600
PF3D7_0832300
PF3D7_1102500
PF3D7_0801900
PF3D7_0525000
PF3D7_0206400
PF3D7_0206300
PF3D7_0423700
PF3D7_1252500
PF3D7_0215000
PF3D7_1472900
PF3D7_1038700
PF3D7_1134600
PF3D7_1236200
PF3D7_1477700
PF3D7_1302100
PF3D7_0406200
PF3D7_1016500
PF3D7_1473700
PF3D7_0422000
PF3D7_1467600
PF3D7_0113400
PF3D7_1102600
glu-tRNA synt.
pk4
B
Fold change 3D7/F12
ring stages (4-12hpi)
early schizonts (32-40hpi)
late schizonts (40-48hpi)
neg. controls
PF3D7_1302100
PF3D7_1477700
PF3D7_0406200
PF3D7_1222600
PF3D7_1102600
PF3D7_1467600
PF3D7_0423700
PF3D7_1473700
PF3D7_1102500
PF3D7_0215000
PF3D7_1134600
PF3D7_1472900
PF3D7_0113400
PF3D7_0832300
PF3D7_0422000
PF3D7_1016500
PF3D7_1252500
PF3D7_0801900
PF3D7_1038700
PF3D7_1236200
PF3D7_0525000
PF3D7_0206400
PF3D7_0206300
glu-tRNA synt.
pk4

Figure S7. Confirmation of induction of novel early gametocyte genes by qRT-PCR (related to Figure 7D)

(A) qRT-PCR experiments confirm the induction of commitment and early gametocyte genes in PfHP1-depleted parasites. PfHP1 depletion was induced 4-12hpi by Shield-1 removal (3D7/HP1^{OFF}). Values represent the fold change in relative expression in 3D7/HP1^{OFF} compared to 3D7/HP1^{ON} parasites at four consecutive TPs: commitment phase (TP1, 40-48hpi generation 1); gametocyte ring stage (TP2, 4-12hpi generation 2); stage I gametocytes (TPs3 and 4, 24-32hpi and 32-40hpi). Relative expression values were normalised for arginyl tRNA-synthetase (PF3D7_1218600) transcripts. See also Table S1. (B) qRT-PCR experiments confirm the induction of commitment and early gametocyte genes in 3D7 wild type parasites compared to the gametocyte-deficient clone F12. Values represent the fold change in relative expression in 3D7 compared to F12 at three consecutive TPs: ring stages (TP1, 4-12hpi); early schizonts (TP2, 32-40hpi); late schizonts (TP3, 40-48hpi). Relative expression values were normalised for arginyl tRNA-synthetase (PF3D7_1218600) transcripts. See also Table S1.

Table S1. Processed microarray dataset (related to Figures 4, 5, 6, 7, S3 and S7) (not included due to size, but can be found online)

Columns A-C: Basic gene information. Columns D-Y: averaged Cy5/Cy3 log2 ratios for all transcripts and time points (TPs 2-12) harvested from 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites (related to Figures 4, 6A and 7A). Columns Z-AA: Phase calculation values (see Supplemental Experimental Procedures) (related to Figure 4). Columns AB-AD: Classification of genes into heterochromatic, euchromatic PfHP1-associated, and euchromatic groups based on previously published data (Flueck et al., 2009) and manual curation (related to Figure 5). Columns AE-AG: mean fold change in gene expression (TPs 4-9) in 3D7/HP1^{OFF} compared to 3D7/HP1^{ON} parasites (column AE), SAM significance values (% FDR) (column AF), and significantly de-regulated genes (U, up-regulated; D, down-regulated) (column AG) (related to Figures 5 and S3). Columns AH-AM: mean fold change in gene expression in 3D7/HP1^{OFF} compared to 3D7/HP1^{ON} parasites and SAM significance values (% FDR) for TPs 7-12 (columns AH and AI), TPs 7-9 (columns AJ and AK), and TPs 10-12 (columns AL and AM) (related to Figure 7D). Columns AN-AS: genes expressed in gametocytes based on published work (Silvestrini et al., 2010; Silvestrini et al., 2005; Young et al., 2005; Eksi et al., 2012; Eksi et al., 2005) (related to Figures 7C, 7D, and S7). Column AT: early gametocyte genes predicted in this study (see Supplemental Experimental Procedures) (related to Figure 7D).

Table S2: List of all primers used in this study (related to Supplemental Experimental Procedures)

Primers used in targeted ChIP experiments

Primer	Sequence (5'-3')	gene ID and name
upsA fw	gacggctaccacagagacaa	PF3D7_1100200
upsA rev	cgtcatcatcgtcttcgttt	
upsC fw	accgccccatctagtgatag	PF3D7_0412400
upsC rev	cacttggtgatgtggtgtca	
upsB fw	tgacgactcctcagacgaag	PF3D7_0426000
upsB rev	ctccactgacggatctgttg	
clag3.1 fw	tctagtaatgagaattagttggaca	PF3D7_0302500
clag3.1 rev	ataaatatttggatgcttcagcag	
clag3.2 fw	tctagtaatgagaattagttggaca	PF3D7_0302200
clag3.2 rev	gaacaaatatgtttctgaactagga	
ApiAP2 fw	tggtggtaataagaacaacagaggt	PF3D7_1222600

ApiAP2 rev	ccatcataatcttcttcttcgtcg	
arginyl fw	aagagatgcatgttggtcattt	PF3D7_1218600
arginyl rev	gagtaccccaatcacctaca	
actin fw	agcagcaggaatccacaca	PF3D7_1246200
actin rev	tgatggtgcaagggttgtaa	

Primers used in quantitative RT-PCR

	Sequence (5'-3')	**gene ID and name**
Pfs16 fw	agttcttcaggtgcctctcttca	PF3D7_0406200
Pfs16 rev	agctagctgagtttctaaaggca	
Pfg27 fw	cttagcaaggatcctgagaagttt	PF3D7_1302100
Pfg27 rev	gttgacaatgttatcttggacacgt	
PK4 fw	ctcatattccatacgatgctccat	PF3D7_0628200
PK4 rev	taaactgaaccaaatcctccctgt	
arginyl fw	aagagatgcatgttggtcattt	PF3D7_1218600
arginyl rev	gagtaccccaatcacctaca	
glutaminyl fw	tggctaggatatgattggaaagaaca	PF3D7_1331700
glutaminyl rev	tacggttctatttctatatggtgaatca	
PF11_0037 fw	aacactagagaacatggaggtgaa	PF3D7_1102500
PF11_0037 rev	ttctaacattgcttctactcatacca	
PF11_0357 fw	agaaatctcttccccgaaggaac	PF3D7_1134600
PF11_0357 rev	cagggtacatggtctaaatgtcc	
PF14_0706 fw	agtgctgttgtaggtattgttgtga	PF3D7_1473700
PF14_0706 rev	tgcccataaaatctgaaatatcccat	
PFB0685c fw	gattaactgaaacgactggacca	PF3D7_0215000
PFB0685c rev	cctcttggtaaactatcctttgca	
PF14_0644 fw	tcacgaaaggaaatgtatagaggaaa	PF3D7_1467600
PF14_0644 rev	gttctctattgatgactcttcttggt	
PF11_0038 fw	tggtggattggcacttatacctttt	PF3D7_1102600
PF11_0038 rev	cattggtataccacataagattcctc	
PFD1120c fw	tccaggatttatgaacgtattaggga	PF3D7_0423700
PFD1120c rev	acctaagagtaaagcaagaccagt	
PF14_0697 fw	ccaatagctgatgacatgcactg	PF3D7_1472900
PF14_0697 rev	gcatcactaaaacacgattacagc	
PFL1085w fw	tggtggtaataagaacaacagaggt	PF3D7_1222600
PFL1085w rev	ccatcataatcttcttcttcgtcg	
PFD1035w fw	tattcaagttcagtgccatgttcc	PF3D7_0422000
PFD1035w rev	gaatagcacatttagcatagatatcaga	
PFL1750c fw	gatgacgtatggtctaacgacga	PF3D7_1236200
PFL1750c rev	taacccttttcgagtgctctatgg	
PFL2525c fw	gacaacgaacaatccttaggtacat	PF3D7_1252500
PFL2525c rev	tcttcggaacagaacgtcttaatttg	
MAL7P1.224 fw	gtgttgttgcactaagaaagaacttg	PF3D7_0832300
MAL7P1.224 rev	ttaccaattcgtatgctacatctgta	
MAL8P1.154 fw	tacctacacaagagacacaaacaaat	PF3D7_0801900
MAL8P1.154 rev	tttctgctgctttcttaaatcgctta	
PF10_0161 fw	tgtagatgaaatggcatgtggtac	PF3D7_1016500
PF10_0161 rev	ttcacagttctcatcactagtctct	
PFA0640c fw	acaacaacccaatcagatatacaact	PF3D7_0113400
PFA0640c rev	cgtcctcttcattatcgtcataagt	
PF10_0377 fw	acagtcactatacccaacaacattc	PF3D7_1038700
PF10_0377 rev	atctagtggttgttcaatatgataagaa	
PFB0279w fw	ccttatcagaaatgcttagtttctca	PF3D7_0206300
PFB0279w rev	cgtcaatgttataatgttcttctgtta	
PFB0280w fw	aagtgataaggagaatgatcgtcc	PF3D7_0206400
PFB0280w rev	tctgtgtttgttatggttacctcg	
PFE1245w fw	caacagcatgaacagcagtatgaa	PF3D7_0525000

PFE1245w rev	tctgagaattcgtgctgacattac	
PF14_0748 fw	ccagtgttagaattacgagtgct	PF3D7_1477700
PF14_0748 rev	tcagctttatcagaattcccattgt	

Primers used PCR

	Sequence (5'-3')	**gene ID and name**
hp13'rep fw - XhoI	aact**ctgcag**aaaaaatgaagaaaaagatggaaagcttaaaaac	PF3D7_1220900
hp13'rep rev - NotI	cat**ggcggccgc**cagctgtacggtatcttagtct	
southern probe fw	cagtccatggattgactttaatatttaaatgtcc	PF3D7_1220900
southern probe rev	cagtgcggccgctttttatcgaaagctaatgagac	
hp1 fl fw - BamHI	gatc**ggatcc**aatgacagggtcagatgaag	PF3D7_1220900
hp1 fl rev - NcoI	gatc**ccatgg**ataagctgtacggtatcttag	
DD fw - SpeI	gact**actagt**atgggagtgcaggtggaaacc	
DD rev - SalI	gactt**gtcgac**tcattccagttttagaagctccac	
linker DD fw - BamHI,NheI	gttt**ggatcc**tgt**gctagc**tatggc	
linker DD rev - NotI,NheI,BamHI, PstI	**ggccgc**cata**gctagc**aca**ggatcc**aaa**ctgca**	

2.12 Supplemental Experimental Procedures

2.12.1 Transfection constructs

pH-GFP was generated by excision of the fragment encoding the rep20 repeat region and calmodulin promoter in pHcamGFP (Witmer et al., 2012) with *Pst*I/*Not*I, followed by replacement with a *Pst*I-*BamH*I-*Nhe*I-*Not*I linker sequence. The linker was obtained by annealing complementary oligonucleotides linker-DD-F and linker-DD-R. pH-GFP-DD was then obtained by inserting a PCR fragment encoding the destabilisation domain (DD) (amplified from pJDD41 (kind gift from M. Duraisingh) (Dvorin et al., 2010) downstream of and in-frame with *gfp* into *Spe*I/*Sal*I-digested pH-GFP. The 3' replacement vectors pHP1-GFP and pHP1-GFP-DD were generated by inserting a 645bp fragment corresponding to the 3' end of *pfhp1* into *Pst*I/*Not*I-digested pH-GFP or pH-GFP-DD, respectively. All primer sequences are listed in Table S2.

2.12.2 Production and affinity purification of rabbit α-PfHP1 antibodies

Full-length *pfhp1* was cloned into pET-24d(+) (Novagen) and expressed in BL21(DE3) pMICO *Escherichia coli* (Cinquin et al., 2001) as C-terminally 6xHis-tagged fusion (PfHP1-cHis). PfHP1-cHIS was purified on Ni^{2+}-agarose (Sigma-Aldrich) and eluted with 200mM imidazole-HCl in buffer L (50mM Tris-HCl, 500mM NaCl, 25% (w/v) glycerol, pH 7.5). Approx. 2.5mg of purified protein was precipitated with TCA and used for immunisation of two rabbits. 4ml each of the final bleeds were diluted 1:5 in buffer A (20mM NaH_2PO_4-NaOH, pH 7.0) and IgG was bound to 5ml HitrapProtein A HP columns (GE Healthcare), washed with 20 column volumes (CV) of buffer A and eluted in 0.1M trisodium citrate-HCl, pH 3.0.

The eluates were neutralised by addition of 350µl 1M Tris-HCl (pH 9.0) per ml elution, followed by buffer exchange to PBS using HiTrap Desalting columns (GE Healthcare). The purified fractions contained 2-3mg/ml IgG.

N-terminally 6xHis-tagged PfHP1 (nHis-PfHP1) was used for affinity purification of α-PfHP1 antibodies from both rabbit sera using a method modified from Gu and colleagues (Gu et al., 1994). nHis-PfHP1 was produced by cloning full length *pfhp1* into modified pET-41a(+) (Novagen) where the sequence encoding the N-terminal GST and S-tags was replaced by a linker encoding a 6xHis tag (MHHHHHHSS). nHis-PfHP1 was expressed in BL21-CodonPlus (DE3)-RIl cells (Stratagene) at 20°C by auto-induction in ZYM5052 medium (Studier, 2005), and immobilised on a Ni^{2+}-charged HiTrap IMAC FF column (GE Healthcare).

6ml of purifed IgG fraction was adjusted to 2% TritonX-100, 20mM imidazole-HCl, 300mM NaCl, pH 7.4. Antibodies were loaded onto the nHis-PfHP1 column at a flow rate of 0.2ml/min. The column was washed at 1ml/min with 20 CV buffer T (50mM Tris-HCl, 300mM NaCl, 20mM imidazole-HCl, 2% Triton-X100, pH 7.4) followed by 20 CV buffer 1 (50mM Tris-HCl, 1M NaCl, pH 7.4). α-PfHP1 antibodies were eluted by 4 CV 4M $MgCl_2$ at 0.3ml/min. The eluate was buffer-exchanged to TBS (50mM Tris-HCl, 150mM NaCl, pH 7.4) using HiTrap Desalting columns. Finally, antibodies were buffer-exchanged and concentrated to 0.1-0.2mg/ml in 16mM Na_2HPO_4, 4mM NaH_2PO_4, 150mM NaCl, 25% glycerol using Amicon Ultra centrifugal filters with a 10kDa cut-off (Millipore) and stabilised by addition of NaN_3 to 2mM. The affinity-purified α-PfHP1 antibodies were tested and validated against recombinant PfHP1 (data not shown) and endogenous PfHP1 and PfHP1-GFP-DD by Western blot (Figure S1).

2.12.3 Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA was isolated using Ribozol (Amresco) according to the manufacturer's manual and further purified using the RNeasy Plus Mini Kit (Qiagen) for removal of gDNA. Residual gDNA was digested with TURBO DNA-*free* DNAseI (Ambion). All samples were tested negative for contaminating gDNA by qPCR. RNA was reverse transcribed using the RETROscript Kit (Ambion). qPCR reactions for absolute transcript quantification were performed at final primer concentrations of 0.4µM using SYBR Green Master Mix (Applied Biosystems) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) in a reaction volume of 12µl. All reactions were run in triplicate yielding virtually identical Ct values. Serial gDNA dilutions were used as standards for absolute quantification. Cycling conditions were: 50°C, 2min; 95°C 10min, followed by 40 cycles of 95°C, 15sec; 60°C 1min. Product-specific amplification was ensured by melting curve analysis for each reaction. Relative transcript

levels were calculated by normalisation against the house-keeping gene arginyl-tRNA synthetase (PF3D7_1218600) (Frank et al., 2006). Primer sequences are listed in Table S2.

2.12.4 Targeted Chromatin Immunoprecipitation (ChIP-qPCR)

3D7/HP1^{ON} parasites were synchronised twice 16 hours apart to obtain an eight hour growth window and then split into two populations, one of which was taken off Shield-1 at 4-12hpi. Sample pairs were harvested at 16-24hpi and 40-48hpi in generation 1, and at 16-24hpi in generation 2. After crosslinking with formaldehyde, parasites were released from iRBCs by saponin treatment and nuclei were isolated by parasite lysis in CLB (20mM HEPES (pH7.9), 10mM KCl, 1mM EDTA, 1mM EGTA, 0.65% NP40, 1mM DTT, 1x protease inhibitors (Roche Diagnostics)). Nuclei were washed, resuspended in 150µl ($5x10^6$ nuclei/µl) sonication buffer (1% SDS, 50mM Tris-HCl (pH8), 10mM EDTA, protease inhibitor (Roche Diagnostics)) and sheared by sonication in a Bioruptor UCD-200 (Diagenode) for 15min at 30sec intervals using high power settings. Aliquots containing 4–5µg DNA were incubated with 0.6µg affinity-purified α-PfHP1, 5µg α-H3K9me3 (Millipore 07_442), or 5µg rabbit IgG negative control antibodies (Millipore 12–370) and bound to protein A beads at 4°C overnight. Beads were washed six times and immunoprecipitated chromatin was eluted with 1% SDS, 0.1M $NaHCO_3$. After de-crosslinking at 65°C overnight DNA fragments were purified using PCR purification columns (Qiagen). qPCR was carried out using the primer sets described in Table S2. The amount of target DNA recovered after immunoprecipitation was directly compared to a ten-fold dilution series of input DNA, and defined as percentage of DNA present in the input sample.

2.12.5 Isothermal Microcalorimetry

Isothermal microcalorimetry and data analysis were performed as originally described for asynchronous parasite cultures using a Thermal Activity Monitor (Model 3102 TAM III, TA Instruments, New Castle, DE, USA) (Wenzler et al., 2012). For monitoring of the heat flow throughout the IDC the following optimisations had to be applied. The sample volume was increased to 3ml to enhance heat flow signal when using low starting parasitaemias. In addition, adjusting the parasitaemia of synchronised cultures depending on IDC stage (see below) before introducing samples into the microcalorimeter facilitated monitoring of a typical heat emission profile across two generations. The heat emission profile of synchronised parasite cultures is characterised by a continuous increase in heat flow during the trophozoite stage until reaching a peak during schizogony, followed by a decrease towards the end of schizogony (unpublished). According to these improvements, 3D7/HP1^{ON} clones were split at 4-12hpi and cultured either in presence or absence of Shield-1. Air-sealed ampoules containing 3ml cultures (5% haematocrit) were prepared in triplicate and loaded at

6-14hpi (0.25% parasitaemia) and 28-36hpi (0.15% parasitaemia) for measurements in generation 1 and 2, respectively.

2.12.6 Microarray Experiments and Data Analysis

Microarray re-annotation. All 10416 oligonucleotide probes on the array were remapped to genome sequence released on PlasmoDBv9.0 (www.plasmodb.org) using NCBI Blast 2.2 with an E-value cut-off 10^{-1}. In total, 9985 probes representing 5232 coding genes had unique hits with high first hit bit scores >100 and low second hit bit scores <56. Only these probes were considered for further analysis.

Microarray data processing and analysis. All data were processed using R and Perl language and Microsoft Excel. Data normalisation was done using Limma package of R (Smyth, 2005). Briefly, log2 ratios of Cy5/Cy3 signal intensities were calculated for each spot after 'normexp' background correction and normalised by locally estimated scatterplot smoothing (LOESS) within arrays and quantile-normalised between arrays. Flagged spots and spots with both Cy3 and Cy5 intensities lower than 1.5-fold the corresponding microarray background were discarded. Log2 ratios for multiple probes per gene were averaged. 5133 coding genes showed expression values at a minimum of four and five time points in generation 1 and 2, respectively (genes with more than one missing data in either generation 1 or 2 were discarded). For missing expression values, a k-nearest neighbour (KNN) imputation method was applied using the impute package of R (estimated values were less than 0.3% of the total). The averaged relative expression values for each gene (Cy5/Cy3 log2 ratios) are presented in Table S1.

Sine regression. We modelled the expression profile for each gene using sine function according to the formula: $E(t) = A\sin[\omega (t-\alpha)] + C$, where E(t) is the log2 ratio at the t hour of hpi; A is the amplitude of the expression profile across the IDC; C is the vertical offset of profile from zero; ω is the angular frequency given by $\omega = 2\pi/48$; and α is the horizontal offset of profile from zero. We used modified α as the phaseogram to indicate gene expression timing where genes were sorted according to the phase from $-\pi$ to π across all the time points. In addition, an F-test p-value was calculated for each sine regression to indicate how significantly the expression data fitted to the regressed sine curve. We named it sine-fit p-value and defined a cut-off <0.01 for significantly regular changing of mRNA abundance across the IDC.

Mapping test samples to a high resolution reference IDC transcriptome. To estimate the best-fit IDC phase for each RNA sample we mapped each experimental transcriptome to a reference time point in a 3D7 IDC transcriptome dataset according to previously described approaches (Foth et al., 2011; Mok et al., 2011). The reference IDC dataset was determined

at 2 hour resolution by spline interpolation on an IDC time course experiment sampled in 8 hour intervals (unpublished). For age mapping, we only considered ~3100 genes displaying profound and regular mRNA abundance changes across the IDC. These genes were defined by a sine-fit p-value cut-off (<0.01) and maximum fold change of mRNA abundance cut-off (≥2). Spearman rank correlation coefficients (ρ) between global mRNA profiles were calculated for each experimental and reference time point. The reference time point with the highest ρ value was assigned as the best-fit estimated IDC time point for each experimental test sample (Figure 4).

Identification of differentially expressed genes. We performed paired two-class Significance Analysis of Microarrays (SAM) (Tusher et al., 2001) to identify genes with significant differential expression between 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites (q-value (fdr) cut-off <0.1; mean fold change cut-off ≥1.5). Heatmaps were generated using Gene Cluster 3.0 (Eisen et al., 1998) and the Java Treeview program (Saldanha, 2004). Scatterplots were generated using Microsoft Excel.

Identification of early gametocyte genes. Using the transcriptional profiles of the five well-established early gametocyte markers *pfs16* (PF3D7_0406200) (Bruce et al., 1994), *pfg27* (PF3D7_1302100) (Alano et al., 1991), *pfg14_744* (PF3D7_14777300), *pfg14_748* (PF3D7_1477700) (Eksi et al., 2005) and *etramp10.3* (PF3D7_1016900) (Silvestrini et al., 2005) we defined simple and stringent filtering criteria to search for other genes potentially involved in sexual commitment and early gametocyte development. Predicted genes were required to display (i) positive log2 ratios in 3D7/HP1^{OFF} parasites at each TP harvested after re-invasion (TPs 7-12); (ii) >2-fold increased mean expression (fdr<0.1) in 3D7/HP1^{OFF} compared to 3D7/HP1^{ON} parasites after re-invasion (TPs 7-12); and (iii) >4-fold increased mean expression (fdr<0.1) in 3D7/HP1^{OFF} compared to 3D7/HP1^{ON} parasites either in ring stages (TPs 7-9) or stage I gametocytes (TPs 10-12). Heterochromatic genes were excluded from the search.

2.13 Supplemental References

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Chapter 3: Functional characterisation of the chromosome-end associated protein PfSIP2 in *Plasmodium falciparum*

Working paper

The results presented in this chapter will be part of a future manuscript detailing the structure and function of PfSIP2 (collaborative project with Igor Niederwieser (Voss Lab, Swiss TPH) and Richard Bartfai (RIMLS, Radboud University Nijmegen, The Netherlands) on which I will be listed as second author.

I performed all experiments and analyses related to this chapter except for the ChIP-Seq experiments, which were performed in Richard Bartfai's lab (RIMLS, Radboud University Nijmegen, The Netherlands).

3.1 Introduction

Regulating gene expression precisely is key for organisms to develop and for cells to differentiate as well as to respond to environmental changes. In all eukaryotes, gene expression is controlled by a complex interplay of transcriptional, post-transcriptional and (post-) translational mechanisms.

Generally, transcription of protein-encoding genes is regulated by a highly conserved transcriptional machinery composed of an RNA polymerase II complex and basal transcription factors (TFs). While the latter bind to the core promoter and are required for baseline activity of a given gene, specific TFs bind to regulatory promoter elements to fine-tune transcription (Roeder, 2003). On the level of chromatin, epigenetic mechanisms are able to coordinate the overall activity state of large chromosomal regions, but are also capable of controlling expression at defined genomic loci.

Plasmodium parasites alter the expression of most genes in a precisely timed and tightly controlled manner (Bozdech et al., 2003; Le Roch et al., 2003, 2004). In spite of recent advances in the genetics and epigenetics fields, however, the corresponding regulatory mechanisms remain poorly understood. Although functional regulatory sequence motifs in promoter regions have indeed been identified for some genes, these sequence motifs have no known counterparts in model eukaryotes, where these processes have been studied in great detail (Horrocks et al., 2009). Furthermore, in contrast to the presence of a conserved RNA polymerase II complex and a set of basal TFs in *Plasmodium* parasites, genome analyses yet failed to identify specific TFs found in other eukaryotes (Callebaut et al., 2005; Coulson and Ouzounis, 2003; Coulson et al., 2004). The paucity of recognisable specific TFs is puzzling and stands in great contrast with the tightly regulated cascade of gene expression that controls the complex development of the malaria parasite within the human host and the mosquito vector.

In 2005, an extensive and highly sensitive bioinformatics approach, using sequence similarities of known eukaryotic DNA-binding domains, revealed a new family of TFs in the Apicomplexan phylum (Balaji et al., 2005). The newly identified family was termed Apicomplexa AP2 (ApiAP2), since their predicted DNA-binding domains are related to those found in plant Apetala 2 (AP2) DNA-binding proteins. Interestingly, these AP2 proteins are unique to the plant lineage and in *Arabidopsis thaliana* they represent the second largest class of transcription factors and drive both enhancing as well as repressing activities in transcriptional regulation (Riechmann et al., 2000).

In *Plasmodium falciparum,* 27 ApiAP2 family members have been identified so far and most of their AP2 domains show high sequence conservation across *Plasmodium* species (Balaji

et al., 2005). Each member is characterised by at least one copy of an AP2-like DNA-binding domain and most of them show unique sequence-specific DNA-binding preferences *in vitro* (Campbell et al., 2010; De Silva et al., 2008).

Strikingly, 21 of the 27 members show a tightly controlled temporal expression pattern during the intra-erythrocytic development cycle (IDC) and are expressed in either ring, trophozoite or schizont stages (Balaji et al., 2005; Bozdech et al., 2003; Campbell et al., 2010; Le Roch et al., 2003). While most ApiAP2 factors are active during the asexual blood stage, several ApiAP2 members were shown to be actively transcribed also in sexual parasites as well as in liver stages and insect vector stages (reviewed in Painter et al., 2011). These characteristics render the group of ApiAP2 TFs excellent candidate regulators for the cell cycle- and life cycle-specific transcription of genes that is so prominently observed in the malaria parasite throughout its life cycle.

So far, however, only little is known about how ApiAP2 factors regulate stage-specific transcription. The following paragraph summarises recent achievements in the characterisation of *Plasmodium* ApiAP2 TFs that were indeed found to carry out essential roles in transcriptional regulation of genes specific to distinct developmental stages of the malaria parasite.

Regulatory activity of an ApiAP2 protein was first described by Yuda et al. who discovered intriguing roles of the *P. berghei* ApiAP2 protein AP2-O (PBANKA_090590) in ookinete development (Yuda et al., 2009). Yuda and colleagues showed that AP2-O is released from translational repression in ookinetes and binds to *cis*-acting elements in promoter regions of ookinete-specific genes to activate their transcription. Intriguingly, recent ChIP-Seq data revealed that the complete gene set used to regulate the processes of ookinete morphogenesis, locomotion, mosquito midgut-invasion and oocyst development, is under the control of AP2-O (Kaneko et al., 2015). Another study showed that the ApiAP2 protein AP2-Sp (PBANKA_132980) regulates *P. berghei* sporozoite development in the *Anopheles* vector. AP2-Sp interacts with unique *cis*-elements in promoter regions of sporozoite-specific genes to activate their transcription (Yuda et al., 2010). Similarly, AP2-L (PBANKA_021440) regulates genes with essential functions for liver stage development of *P. berghei* (Iwanaga et al., 2012). More recently, two independent studies in *P. falciparum* and *P. berghei* showed that the ApiAP2 protein AP2-G (PF3D7_1222600; PBANKA_143750) is a master regulator of sexual conversion in malaria parasites (Kafsack et al., 2014; Sinha et al., 2014). Interestingly, AP2-G was subsequently found to be controlled by epigenetic means (Brancucci and Bertschi et al., 2014, Chapter 2 this thesis; Coleman et al., 2014).

So far, the characterisation of the *P. falciparum* SPE2-interacting protein PfSIP2 (PF3D7_0604100) represents the only study detailing the role of an ApiAP2 protein active in

asexual blood stage parasites (Flueck et al., 2010). This study showed that PfSIP2 is actively transcribed after the onset of the S-phase in late intra-erythrocytic parasites, and the authors suggest that full-length PfSIP2 is processed to generate a functionally active N-terminal fragment. This fragment contains two adjacent AP2 domains that interact specifically with the highly conserved subtelomeric *var* gene promoter element 2 (SPE2) *in vitro* and *in vivo* (Flueck et al., 2010; Voss et al., 2003). This bipartite DNA motif occurs in tandem arrays at conserved positions approximately 2kb upstream of *ups*B-type *var* genes as well as within the telomere-associated repetitive elements 2 and 3 (TARE2/3). ChIP data revealed that the PfSIP2 N-terminal fragment is indeed specifically enriched at these subtelomeric SPE2 arrays and co-localises with heterochromatin protein 1 (PfHP1) at chromosome-end clusters (Flueck et al., 2009, 2010). In addition, functional promoter studies suggested that PfSIP2 may contribute to *ups*B promoter-mediated *var* gene silencing, even though overexpression of the PfSIP2 N-terminal fragment did not result in any transcriptional changes in *var* expression. The study further showed that the *pfsip2* locus is refractory to genetic deletion suggesting an essential role for PfSIP2 in parasite survival (Flueck et al., 2010). Due to its localisation and expression profile, the authors suggested that PfSIP2 may play a role in the nucleation of subtelomeric heterochromatin in newly replicating parasites, as well as in chromosome replication and/or segregation (Flueck et al., 2010). These assumptions are backed up by recent work supporting the idea that PfSIP2 may have a leading role in transcribing subtelomeric SPE2 arrays into long non-coding RNA (lncRNAs) (Broadbent et al., 2011). These lncRNAs are thought to play a crucial role in the assembly of subtelomeric heterochromatin and in the maintenance of genome integrity (Broadbent et al., 2011).

The exact function of PfSIP2 in malaria parasites, however, remains unknown. Here, we aim at elucidating PfSIP2 function by (1) mapping endogenous PfSIP2 occupancy at a high resolution using ChIP-Seq; and by (2) generating and characterising an inducible PfSIP2 loss-of-function mutant. Dissecting the function of this protein may be key to understanding mechanisms underlying variable virulence gene expression and genome maintenance in the malaria parasite.

3.2 Results

3.2.1 PfSIP2 expression is tightly regulated during late stage parasites

PfSIP2 localises to chromosome-end clusters and is only temporary expressed in early and late schizont stages (Flueck et al., 2010). In order to study the expression profile of endogenous PfSIP2 in asexual parasites in more detail, we created a transgenic 3D7 cell line where the *pfsip2* 3' end is fused to a GFP-tag (3D7/SIP2^{GFP}). Successful tagging of the endogenous locus was confirmed by Southern blot analysis (Figure 1). Fluorescent tags

allow for visualisation of protein expression in real time in a non-invasive way by live cell imaging. Here, we used GFP since it was found to be nontoxic for malaria parasites and is not likely to interfere with protein function (Tilley et al., 2007).

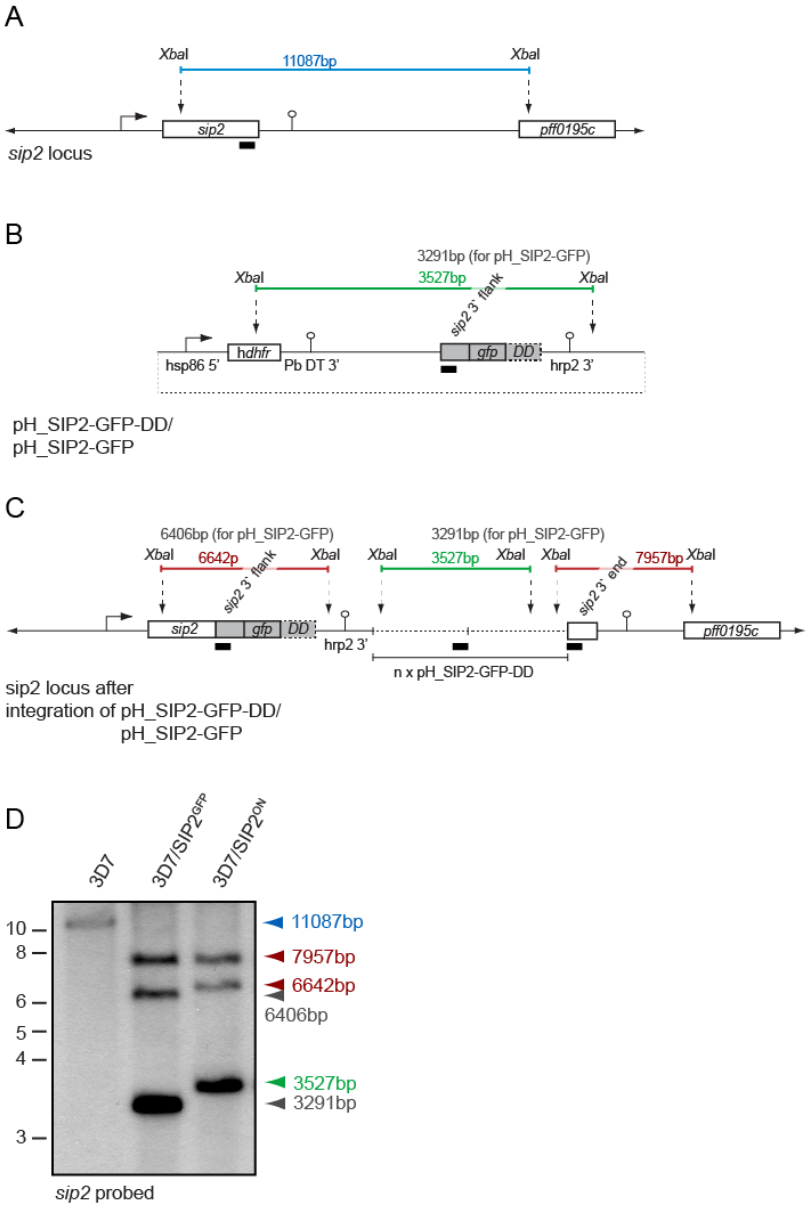

Figure 1. Southern Blot analysis reveals successful integration of plasmid DNA into the endogenous *pfsip2* locus

(A) The endogenous *pfsip2* locus (PF3D7_0604100) is schematically depicted. (B) Schematic display of the vector pH_SIP2-GFP (control) and pH_SIP2-GFP-DD both containing the *pfsip2* 3' end. (C) The endogenous *pfsip2* locus is depicted schematically after plasmid integration of the pH_SIP2-GFP and pH_SIP2-GFP-DD plasmids in 3D7/SIP2^{GFP} and 3D7/SIP2^{ON} parasites, respectively. *Xba*I restriction sites were used for Southern blot analysis and the resulting fragment lengths are indicated in colour. The hybridisation probe is shown as a black solid line. Dashed lines indicate integration of plasmid concatamers. *hsp86* 5', heat shock protein 86 gene promoter; *hdhfr,* human dihydrofolate reductase; Pb DT 3', *P. berghei* dhfr-thymidilate synthase gene terminator; *gfp*, green fluorescent protein; *dd*, destabilisation domain; hrp2 3', histidine-rich protein 2 gene terminator. (D) Southern blotting reveals

successful plasmid integration into the endogenous *pfsip2* locus in 3D7/SIP2^{GFP} parasites and clonal 3D7/SIP2^{ON} parasites. 3D7 wild-type gDNA was used as a control. gDNA was digested with *Xba*I and transferred to a membrane and hybridised with a ^{32}P-dATP-labelled *pfsip2* DNA probe.

To study the expression profile of endogenous PfSIP2 across the IDC, 3D7/SIP2^{GFP} parasites were tightly synchronised to a 6hrs time window and live cell imaging was performed at five consecutive time points (24-30hpi, 30-36hpi, 36-42hpi, 42-48hpi, 6-12hpi). As previously reported, no signal was detected in parasites younger than 36hrs post invasion. However, distinct perinuclear foci could be detected in parasites undergoing schizogony (36-42hpi). This localisation pattern is expected for PfSIP2 bound to target loci at chromosome-end clusters (Flueck et al., 2010). Notably, PfSIP2 was still present in late stage parasites (42-48hpi), but vanished in segmented parasites ready to release new merozoites and was also no longer detectable after reinvasion (6-12hpi) (Figure 2A). This tight expression pattern was corroborated by Western blot analysis of whole cell protein lysates at five consecutive time points (24-30hpi, 30-36hpi, 36-42hpi, 42-48hpi, 6-12hpi) using an anti-GFP antibody for signal detection (Figure 2B). Since PfHP1 is still detectable in ring stage parasites, whereas PfSIP2 is not, this indicates that existing PfSIP2 is actively degraded by the parasite in segmented schizonts and/or merozoites. The 250kDa band (size of PfSIP2 including the GFP-tag) in Western blot further suggests that PfSIP2 is present as full-length protein in whole cell lysates and hence not proteolytically cleaved into an active N-terminal fragment, as stated before (Flueck et al., 2010).

We conclude that PfSIP2 activity is tightly controlled in late stage parasites as the protein can be detected in early schizonts (36hpi) but expression is terminated prior to merozoite formation and the burst of infected erythrocytes (48hpi).

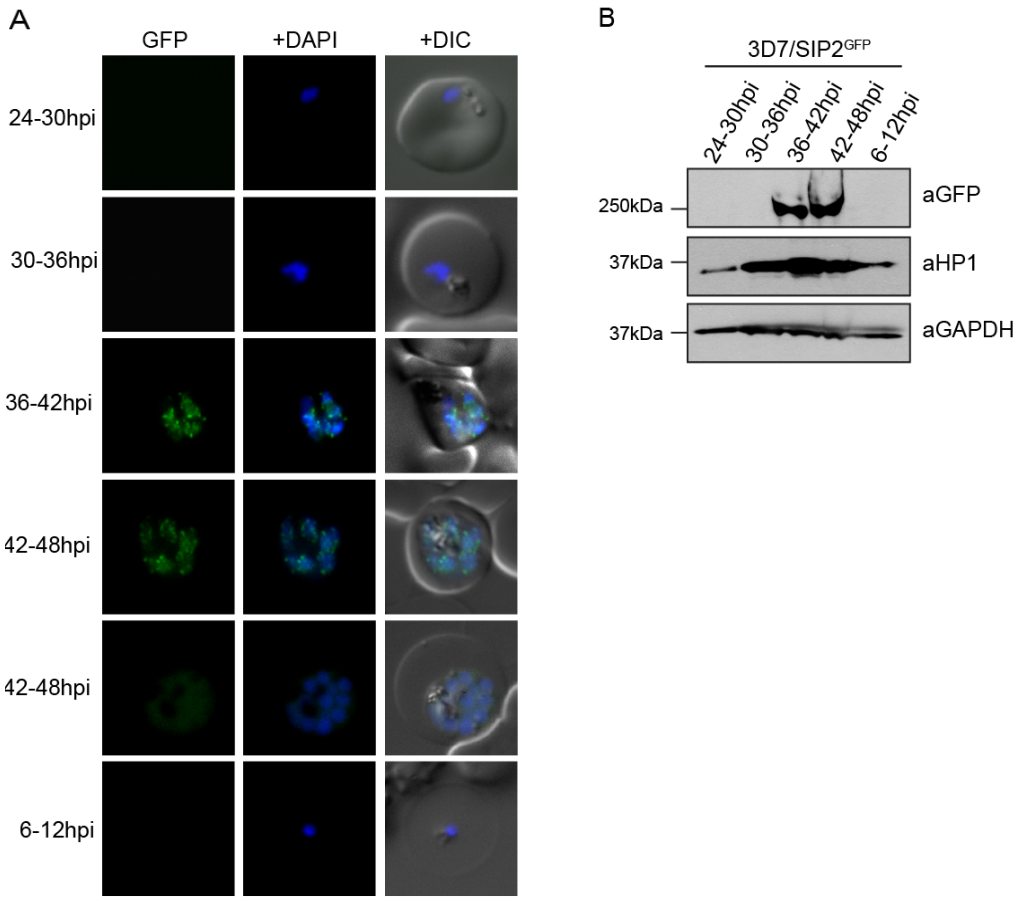

Figure 2. Expression of PfSIP2 is tightly regulated during the asexual blood stage cell cycle

A transgenic cell line that endogenously expresses PfSIP2 with a C-terminal GFP-tag was generated. Parasites were tightly synchronised and analysed at five consecutive time points during the IDC (24-30hpi, 30-36hpi, 36-42hpi, 42-48hpi, 6-12hpi). (A) Live cell imaging revealed that PfSIP2 (green) is only present during a tight time window in schizont parasite stages and localises to chromosome end clusters. Note that segmented parasites ready to release new merozoites at 42-48hpi show reduced levels of PfSIP2. Nuclei were stained with DAPI. DIC, Differential interference contrast. Images were taken with identical settings and for each TP a representative image is shown. (B) Western blot of whole cell extracts corroborates the tight expression profile of full-length PfSIP2 (250kDa including the GFP-tag) in late stage parasites. The Western blot was probed with anti-GFP antibodies. PfHP1 and GAPDH were also probed and serve as expression and loading controls. Same numbers of parasites were loaded.

3.2.2 ChIP-Sequencing confirms genome-wide *in vivo* binding sites of PfSIP2

As described previously, genome-wide ChIP (ChIP-on-chip) suggested that PfSIP2 exclusively binds to the subtelomeric *var* gene promoter element 2 (SPE2) located upstream of *ups*B-type *var* genes as well as within telomere-associated repetitive elements 2 and 3 (TARE2/3) regions (Flueck et al., 2010). However, these data were generated with a transgenic cell line ectopically expressing an N-terminal fragment containing the two ApiAP2 DNA-binding domains. To map the global distribution of endogenous full-length PfSIP2 in *P. falciparum* at a high resolution, we used the 3D7/SIP2^{GFP} cell line to perform ChIP followed

by massive parallel sequencing (ChIP-Seq). To this end, 3D7/SIP2^{GFP} late stage parasites (38-46hpi), expressing endogenous C-terminally GPF-tagged PfSIP2, were treated with formaldehyde to crosslink DNA-protein interactions. Subsequent ChIP experiments followed by in-depth sequencing experiments were performed and analysed by R. Bartfai (RIMLS, Radboud University Nijmegen, The Netherlands). The obtained data confirmed the previously described genomic binding sites of PfSIP2 (not shown, data available on request from R. Bartfai, RIMLS, Radboud University Nijmegen, The Netherlands). Interestingly, since the C-terminal GFP-tag of endogenous PfSIP2 was accessible in ChIP-Seq experiment, we conclude that PfSIP2 is not protelytically cleaved, but rather binds as a full-length protein to the previously described subtelomeric SPE2 arrays.

3.2.3 Generation of an inducible loss-of-function mutant

The role of PfSIP2 in the biology of blood stage parasites is still elusive. Several attempts to generate a PfSIP2 knockout cell line were unsuccessful, suggesting that this protein is essential for asexual blood stage development of the parasite (Flueck et al., 2010). Aiming at the functional characterisation of PfSIP2, we generated an inducible loss-of-function mutant by fusing a GFP-tag and a FKBP-destabilisation domain (DD) to the C-terminus of endogenous PfSIP2 (3D7/SIP2^{ON}) (Figure 1). This well-established approach allows for targeted protein degradation upon removal of the stabilising compound Shield-1 from the culture medium (Armstrong and Goldberg, 2007; Banaszynski et al., 2006). Tagging the protein with GFP further allows one to observe localisation of the GFP-DD fusion protein and to monitor protein degradation after withdrawal of Shield-1. Note also that we have used this strategy successfully to establish a conditional PfHP1 loss-of-function mutant (Brancucci and Bertschi et al., 2014; Chapter 2, this thesis).

A clonal 3D7/SIP2^{ON} cell line was obtained through limiting dilution. Immunofluorescence and Western blot analyses revealed that, in presence of Shield-1, the expression profile and localisation pattern of the GFP-DD fusion protein is indistinguishable from PfSIP2 tagged with GFP only in 3D7/SIP2^{GFP} (Figures 3A and 3B). Notably, 3D7/SIP2^{ON} parasites underwent asexual replication with comparable dynamics as 3D7/SIP2^{GFP} parasites (data not shown).

In order to induce PfSIP2 degradation, Shield-1 was withdrawn from 3D7/SIP2^{ON} parasites at 4-12hpi (3D7/SIP2^{OFF}). Immunofluorescence and Western blot analyses revealed that PfSIP2 is gradually depleted under non-stabilising conditions. Whereas protein levels are only reduced in 3D7/SIP2^{OFF} parasites at 36-42hpi, almost complete degradation can be observed at 40-48hpi (Figures 3A and 3B). Surprisingly, no detectable growth phenotype was observed for PfSIP2-depleted parasites. Neither morphology, nor progression through the

IDC was hampered in 3D7/SIP2^{OFF} cells, even when parasites were cultured in absence of Shield-1 for several generations. Also, we found that PfSIP2-depleted populations displayed identical sexual conversion and asexual multiplication rates as 3D7/SIP2^{ON} parasites and the 3D7/SIP2^{GFP} control cell line (data not shown). Here, it is important to note that monitoring of PfSIP2-GFP-DD degradation by Western blot and IFA experiments, as well as phenotypic studies of 3D7/SIP2^{OFF} parasites was analysed in a total of seven independent clones, and all of them behaved identically, as described above (data not shown).

In summary, the significant reduction of PfSIP2-levels does not compromise parasite growth and does not result in apparent phenotypical changes in parasites developing through the IDC. Nevertheless, this cell line may be an excellent tool to study the potential regulatory role of PfSIP2 in gene transcription.

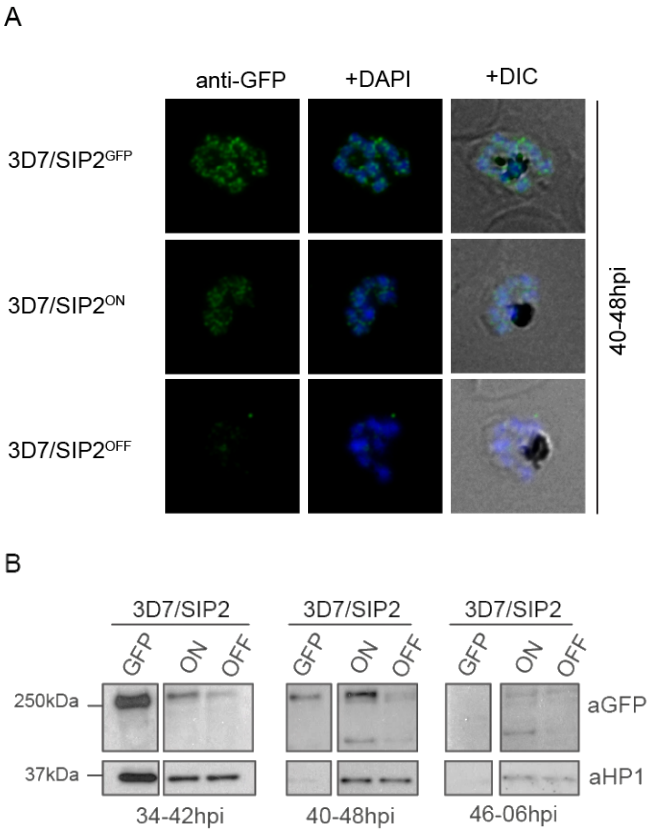

Figure 3. PfSIP2 levels are significantly reduced in the loss-of-function mutant

A conditional loss-of-function mutant was created by fusing endogenous PfSIP2 to a GFP-tag and a FKBP-destabilisation domain. Parasites were split into two different cultures at 4-12hpi and cultured either in presence (3D7/SIP2^{ON}) or in absence of Shield-1 (3D7/SIP2^{OFF}). Localisation and expression levels of the PfSIP2-GFP-DD fusion protein (270kDa) were analysed at the indicated time points. The 3D7/SIP2^{GFP} cell line was used as control. (A) IFA of MeOH-fixed parasites display proper localisation of the PfSIP2-GFP-DD fusion protein (green) in the control parasite and in 3D7/SIP2^{ON}. In contrast, protein levels are significantly reduced in 3D7/SIP2^{OFF} parasites. Images were taken with identical settings and representative images are shown for each condition. Nuclei were stained with DAPI (blue). The localisation of PfSIP2 was visualised using anti-GFP antibody (green). DIC, Differential

interference contrast. (B) Western blot of whole cell lysates of 3D7/SIP2^{ON} and 3D7/SIP2^{OFF} parasites and the control cell line 3D7/SIP2^{GFP} harvested at the three indicated time points (34-42hpi, 40-48hpi, 46-06hpi) corroborates significant depletion of PfSIP2 in 3D7/SIP2^{OFF} parasites. Western blot was probed with anti-GFP antibodies. Equal parasite numbers were loaded. PfHP1 was used as loading control.

3.2.4 Genome-wide transcriptional profiling of PfSIP2-depleted parasites

Here, we conducted transcriptional profiling of the 3D7/SIP2^{ON} cell line to study possible changes in gene regulation upon PfSIP2-depletion. To this end, 3D7/SIP2^{ON} parasites were split at 4-12hpi into two populations and henceforth cultured in presence or absence of Shield-1 for two subsequent generations. In generation 3, RNA of 3D7/SIP2^{ON} and 3D7/SIP2^{OFF} parasites was harvested at four consecutive time points (8-16hpi; 18-26hpi; 28-36hpi; 38-46hpi) covering the entire IDC. Genome-wide transcriptional profiling was performed using an Agilent microarray platform (Kafsack et al., 2012). Data analysis revealed that no genes are significantly differentially expressed between 3D7/SIP2^{ON} and 3D7/SIP2^{OFF} parasites (not shown, data available in the Gene Regulation Laboratory, Swiss TPH, Basel, Switzerland).

3.3 Conclusion and Outlook

In-depth analysis of endogenous PfSIP2 expression levels across the entire IDC revealed a narrow expression window for this protein of approximately 10hrs. Expression initiation correlated with the onset of schizogony in blood stage parasites (36hpi) and terminated in newly formed merozoites ready to be released form the infected red blood cell (48hpi).

Previous data suggested that PfSIP2 undergoes proteolytic cleavage to release an active N-terminal fragment carrying the DNA-binding activity (Flueck et al., 2010). However, here we show that full-length protein is present in whole cell lysates, refuting that PfSIP2 is processed. Moreover, accessibility of the C-terminal GFP-tag of endogenous PfSIP2 in ChIP-Seq experiments reassures that indeed full-length protein binds to subtelomeric SPE2 motifs. While in previous study by Flueck and colleagues, nuclear lysates were prepared under native conditions (Flueck et al., 2010), we analysed whole cell lysates that were prepared under strong denaturing conditions. This hence strongly corroborates that the previously observed proteolytic cleavage is not of true nature, but rather occurred accidentally during preparation of native nuclear protein extracts.

Since PfSIP2 is prone to rapid degradation, we aimed to improve extraction procedures of full-length PfSIP2 under native conditions. To this end, we adapted a protocol using the density gradient compound Iodixanol (Optiprep OptiPrep™ Density Gradient Medium, Sigma Aldrich # D1556) to isolate *P. falciparum* nuclei and separate them rapidly from the cytosolic

compartment (Graham et al., 1994; Niederwieser et al., unpublished). Using this rapid isolation method, we were indeed able to generate native nuclear preparations containing full-length protein (preliminary data, not shown). This technique will be instrumental for conducting future co-immunoprecipitation experiments. Interestingly, previous work identified DNA replication/repair and chromatin remodelling factors as potential PfSIP2-interacting proteins (Flueck et al., 2010). The identification of PfSIP2 interaction partners will be crucial for attempts aiming to dissect and confirm suggested roles of this chromosome-end associated protein in *P. falciparum*. These studies are currently conducted by Igor Niederwieser (Voss lab) using PfSIP2-GFP in Co-IP experiments followed by liquid chromatography tandem mass spectrometry.

Previous data suggested that PfSIP2 is essential for parasite survival (Flueck et al., 2010). Aiming at the functional characterisation of this protein, we successfully established an inducible PfSIP2-knockdown cell line by using the FKBP destabilisation domain technique. Western blot analysis and immunofluorescence assays revealed that expression levels of this protein can indeed be significantly reduced. Surprisingly, however, neither phenotypic changes nor differential gene expression were observed in PfSIP2-depleted parasites. Hence, the inducible FKBP-mediated destabilisation of PfSIP2 did not allow us to gain novel insights into the function of this ApiAP2 protein. One possible explanation for the failure of our attempt is that this system is not acting fast enough to interfere with PfSIP2-functionality. It was shown that the FKBP domain destabilises target proteins within 4 to 6hrs (Armstrong and Goldberg, 2007). Considering that PfSIP2 is expressed only during a very tight time window of approximately 10hrs during the IDC, it is possible that the protein is able to accomplish its function before being degraded. Immunofluorescence assay and Western blot indeed revealed that PfSIP2 was still present in 3D7/SIP2^{OFF} parasites right after the onset of PfSIP2 expression (data not shown) and degradation reached its maximal level only after 6hrs. Moreover, even though protein levels dropped substantially after 6hrs, it is likely that functionally engaged PfSIP2 (i.e. bound to SPE2 elements) is not efficiently targeted to the proteasome for degradation. Remaining pools of the protein may thus allow to fully sustaining the regulatory role of PfSIP2.

Broadbent and colleagues identified lncRNA transcripts covering subtelomeric SPE2 regions (Broadbent et al., 2011). Strikingly, these lncRNA transcripts and PfSIP2 show highly correlated late stage temporal expression profiles, with lncRNA transcripts appearing slightly after PfSIP2 expression (Broadbent et al., 2011). It is hence tempting to speculate that PfSIP2 might be involved in inducing transcription of subtelomeric lncRNAs. These lncRNAs are hypothetically involved in recruiting histone-modifying enzymes that could contribute to the establishment of subtelomeric heterochromatin (Broadbent et al., 2011; Vembar et al., 2014). Due to the lack of time, and since lncRNAs are not represented on the microarray, we

could not yet investigate the potential role of this protein in regulating transcription of SPE2 lncRNAs. Testing this interesting hypothesis by Northern blot, qRT-PCR and/or RNA-Seq analyses using the PfSIP2-loss-of-funtion mutant will be an important future task.

In order to gain insights of the function of PfSIP2, we also suggest to generate a conditional knockout mutant, using the recently established DiCre system (Collins et al., 2013) in combination with the genome-editing tool CRISPR/Cas9 (Ghorbal et al., 2014; Harrison et al., 2014; Yang et al., 2013). On the one hand, CRISPR/Cas9-mediated genome engineering allows for simple and efficient integration of two *loxP* sites flanking the *pfsip2* locus. On the other hand, the DiCre system allows for inducing Cre-recombinase activity in a rapid and highly regulated manner. Such a system will allow for the conditional deletion (floxing) of the entire *loxP*-flanked *pfsip2* locus by site-specific recombination within a single asexual developmental cycle. Floxing is advantageous over FKBP-induced protein degradation since it is capable to rapidly and completely eliminate protein expression on the single cell level and can reach efficiencies of close to 100% in cell populations. These are key features if one aims to study the function of a potentially essential and tightly regulated protein such as PfSIP2. This approach is currently followed up by Igor Niederwieser (Voss lab).

3.4 Experimental procedure

3.4.1 Transfection constructs

To C-terminally tag endogenous PfSIP2 with GFP (and DD), the 3' end of *pfsip2* was amplified from gDNA using the following primer sequences (capital letter refer to restriction enzyme sites cut by *Pst*I and *Not*I)

Forward Primer: ctagCTGCAGaaatatggtgttaacatatacatg
Reverse Primer: ctagGCGGCCGCttttgtctacatgttcatttatct

The *Pst*I/*Not*I digested PCR fragment (700bp) was purified and inserted upstream and in frame with *gfp* into the *Pst*I/*Not*I digested vector pH_GFP and pH_GFP-DD (Brancucci and Bertschi et al., 2014), respectively (Figure 1B).

3.4.2 Parasite Culture and Transfection

P. falciparum 3D7 cell culture was performed according to standard procedures (Trager and Jenson, 1978). Growth synchronization was achieved by repeated Sorbitol treatments (Lambros and Vanderberg, 1979). Transfections were performed as described (Voss et al., 2006) and selected in the presence of 4nM WR99210 (WR) and, if indicated, in presence of

625nM Shield-1. Parasites were cycled 3 weeks on/3 weeks off WR for three times to select for integration of plasmid DNA into the endogenous locus. 3D7/SIP2^{ON} clones were obtained by limiting dilution.

3.4.3 Southern Blot Analysis

Southern blot analysis was performed as described before (Brancucci et al., 2012). Genomic DNA was digested overnight with the restriction enzyme *Xba*I. Fragments were separated on a 0.5xTBE-buffered 0.8% agarose gel and transferred to Hybond N+ Nylon membrane (Amersham). The blot was hybridised at 60°C with a ^{32}P-dATP-labelled *pfsip2* DNA probe (for PCR primer sequences see section 'Transfection constructs'), washed twice for 20min in 2xSSPE 0.1%SDS, and once for 10min in 0.2xSSPE 0.1%SDS, at 60°C.

3.4.4 Western Blot Analysis

Parasite pellets were obtained after saponin treatment of iRBCs and resuspended in 8M Urea, 5%SDS, 40mM Tris pH6.8 and separated on a NuPage 3-8% Tris-acetate gel (Novex). Detection of endogenous SIP2-GFP(-DD) and PfHP1 (loading control) in 3D7/SIP2^{GFP}, 3D7/SIP2^{ON}, 3D7/SIP2^{OFF} parasites was performed using the following primary antibodies and dilutions: mouse anti-GFP (Roche Diagnostics, #11814460001), 1:500; rabbit anti-PfHP1 (Brancucci and Bertschi et al., 2014) 1:5000, mouse anti-GAPDH, 1:10000 (Daubenberger et al., 2003).

3.4.5 Fluorescence Microscopy

Live-cell fluorescence microscopy and immunofluoresence microscopy were performed as described (Witmer et al., 2012). IFAs were performed on methanol-fixed cells using the following primary antibodies and dilutions: mouse anti-GFP (Roche diagnostics, #11814460001) 1:100; rabbit a-PfHP1 (Brancucci and Bertschi et al., 2014), 1:100. Secondary antibody dilutions were as follows: Alexa Fluor 568-conjugated anti-rabbit IgG (Molecular Probes), 1:500; Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes), 1:500. Images were taken at 96-fold magnification on a Leica DM 5000B microscope with a Leica DFC 345 FX camera, acquired via the Leica IM1000 software, and processed using Adobe Photoshop CS6. For each experiment, images were acquired and processed with identical settings.

3.4.6 Microarray Experiments and Data analysis

3D7/SIP2^{ON} parasites were synchronised twice 16hrs apart to obtain an 8hrs age window. At 4-12hpi in generation 1, cultures were split into two populations, one of which was cultured in

absence of Shield-1 (3D7/SIP2^{OFF}). In generation 3, RNA was extracted from 3D7/SIP2^{ON} and 3D7/SIP2^{OFF} at four consecutive time points (8-16hpi, 18-26hpi, 28-36hpi, 38-46hpi) using RiboZol RNA extraction reagent (Amresco). cDNA synthesis and labelling was performed as described (Brancucci and Bertschi et al., 2014). Cy5-labeled test cDNA was mixed with an equal amount Cy3-labeled 3D7 reference cDNA and hybridised onto an Agilent *P. falciparum* microarray (AMADID#037237) as described (Kafsack et al., 2012). Slides were scanned using a GenePix 4000B microarray scanner and analysed using GenePix Pro 7.0 software (Axon Laboratory). Lowess normalization and background elimination was performed using Acuity 4.0 software (Molecular Devices).

3.4.7 Preparation of parasite material for ChIP-Sequencing

3D7/SIP2^{GFP} parasites were synchronised twice 16hrs apart to obtain an 8hrs age window. At 38-46hpi, formaldehyde-crosslinked chromatin was isolated as described (Flueck et al., 2009).

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Chapter 4: Discovery of a novel type of telomere repeat-binding factor in the malaria parasite *P. falciparum*

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I performed all experiments and analyses related to this chapter except for the generation of the 3D7/TRF^{HA} cell line and the ChIP-Seq experiments, which were performed in Richard Bartfai's lab (RIMLS, Radboud University Nijmegen, The Netherlands) and for the expression and purification of recombinant proteins, which was performed by Igor Niederwieser (Voss Lab, Swiss TPH). Further, I prepared illustrations and wrote the manuscript. Till S. Voss edited the manuscript.

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4.1 Abstract

Proper regulation of telomere biology is indispensable for genome maintenance and the survival of all eukaryotic species. The structural and functional integrity of telomeres is safeguarded by a special protein complex known as shelterin/telosome. Telomere repeat-binding factors (TRFs) are conserved components of these complexes and play vital roles in telomere length homeostasis, chromosome end protection and formation of subtelomeric heterochromatin. TRFs bind to double-stranded telomere repeats via a conserved MYB/SANT-type DNA-binding domain and have been described in a wide range of eukaryotes. Here, we report the *de novo* identification of the telomere repeat-binding factor in the malaria parasite *Plasmodium falciparum*, a member of the Alveolate phylum for which TRFs have not been described to date. PfTRF binds to duplex telomeric repeats via a TFIIIA-like zinc finger domain and therefore has a distinct evolutionary origin compared to MYB-type TRFs. *In vivo*, PfTRF binds exclusively to the telomeric tract on all chromosomes and to telomere repeat-like arrays found upstream of a specific subset of subtelomeric virulence genes. Analysis of a conditional loss-of-function mutant showed that PfTRF regulates telomere length homeostasis and is required for efficient cell cycle progression and parasite proliferation. Our findings provide important new insight into the mechanisms regulating genome maintenance and chromosome end biology in *P. falciparum*. They furthermore demonstrate for the first time TRFs emerged by convergent evolution and indicate that malaria parasites employ a divergent molecular complex to preserve telomere function.

4.2 Introduction

Eukaryotes must distinguish telomeres from DNA double-strand breaks (DSB) in order to prevent chromosome-end fusion and deleterious DNA repair events. Furthermore, the inability of the conventional DNA replication machinery to fully replicate the 5’ end of the lagging strand requests processes that restore a net loss of terminal genetic material in continuously dividing cells. These mechanisms are imperative for genome maintenance and are facilitated by the unique genetic and structural adaptations at telomeres (Gilson and Geli, 2007; Fulcher et al., 2014).

Overall telomere architecture is widely conserved across eukaryotes. Telomeric DNA is composed of tracks of short double-stranded (ds) G-rich repeats, terminating in a single-stranded (ss) 3’ overhang. The G-rich overhang serves as template for telomerase reverse transcriptase (TERT), which counteracts the end-replication problem by adding *de novo*-synthesised telomere repeats (Martinez and Blasco, 2015; Nandakumar and Cech, 2013; Greider and Blackburn, 1987). Telomeres are protected by a multimeric protein complex

called shelterin/telosome that safeguards the chromosome end from unwanted DNA repair mechanisms and regulates telomerase-dependent telomere maintenance (de Lange T., 2005; Xin et al., 2008). The specific recruitment of shelterin to telomeres is accomplished by telomere repeat-binding factors (TRFs) that bind to ds telomere repeats in a sequence-specific manner (Xin et al., 2008). TRF homologs have been studied in detail in evolutionary divergent eukaryotes including human TRF1/TRF2 (Broccoli et al., 1997; Zhong et al., 1992), *Schizosaccharomyces pompe* Taz1 (Vassetzky et al., 1999), *Arabidopsis thaliana* TBP1 (Hwang and Cho, 2007), *Trypanosoma brucei* TRF (Li et al., 2005) and *Leishmania amazonensis* TRF (da Silva et al., 2010). In *Saccharomyces cerevisiae*, which lacks a TRF-like protein, RAP1 binds to ds telomere repeats instead (Longtine et al., 1989). Interestingly, despite minimal overall sequence similarity all TRF proteins studied to date as well as ScRAP1 bind to telomere repeats via a MYB/SANT-like DNA-binding domain (Giraud-Panis et al., 2010; Linger and Price, 2009; Bilaud et al., 1996).

Telomere repeat-binding factors play multiple pivotal roles in telomere maintenance. HsTRF1, HsTRF2, SpTaz1 and ScRAP1 are important regulators of telomere length homeostasis and are required to prevent DNA damage responses at telomeres (Cooper et al., 1997; Ferreira and Cooper, 2001; Marcand et al., 1997; Pardo and Marcand, 2005; Smogorzewska et al., 2000; van Steensel B. et al., 1998; van Steensel B. and de Lange T., 1997; Palm and de Lange T., 2008). HsTRF1 and SpTaz1 are essential for efficient DNA replication through the telomeric tract (Miller et al., 2006; Sfeir et al., 2009), and HsTRF2 and TbTRF support G-overhang maintenance (van Steensel B. et al., 1998; Smogorzewska et al., 2002; Li et al., 2005). Furthermore, HsTRF2 and SpTaz1 have been implicated in the formation and stabilisation of higher order protective structures called T-loops, which are believed to form upon invasion of the 3′ G-rich overhang into the ds telomeric tract (Griffith et al., 1999; Stansel et al., 2001; Doksani et al., 2013; Tomaska et al., 2004).

Mutational analyses of ScRAP1, SpTaz1, and HsTRF2 have also uncovered universal roles for TRFs in nucleating and establishing telomeric/subtelomeric heterochromatin, an important structural and functional feature of chromosome ends across species (Benetti et al., 2008; Kanoh et al., 2005; Cooper et al., 1997; Moretti et al., 1994; Grunstein, 1997). Subtelomeric DNA is characterised by densely packed nucleosomes that are enriched in epigenetic marks typically observed for silenced chromatin, including histone hypoacetylation and tri-methylated histone 3 lysine 9 (H3K9me3) and heterochromatin protein 1 (HP1) (or the silent information regulator (SIR) complex in case of *S. cerevisiae* that lacks H3K9me3/HP1) (Tham and Zakian, 2002; Moazed, 2001). Telomeric/subtelomeric heterochromatin is important for proper telomere length control but is most famous for exerting the so-called telomere-position effect (TPE), i.e. the heritable silencing of telomere-proximal gene

expression (Schoeftner and Blasco, 2009; Blasco, 2007; Tham and Zakian, 2002; Ottaviani et al., 2008).

In *Plasmodium falciparum*, the causative agent of the most severe form of malaria in humans, telomere and subtelomere biology plays a fundamental role in parasite survival, virulence and transmission (Hernandez-Rivas et al., 2013; Voss et al., 2014). As unicellular organisms, malaria parasites strictly rely on efficient telomere maintenance mechanisms to secure proliferation capacity throughout their complex life cycle. Furthermore, *P. falciparum* exploits TPE very effectively to drive phenotypic variation, and this has emerged as the parasite's major survival strategy in the human host (Voss et al., 2014). Despite the essential and evolutionary conserved roles for MYB-type TRFs in regulating telomere function, however, such a factor has not been identified and appears to be absent in these parasites. This is intriguing given that the overall structure of *P. falciparum* telomeres is highly similar to that observed in other eukaryotes possessing MYB-type TRFs and that *P. falciparum* telomeres are also maintained by telomerase (Hernandez-Rivas et al., 2013; Figueiredo and Scherf, 2005; Scherf et al., 2001).

P. falciparum telomeres consist of degenerate TT(T/C)AGGG repeats, have a mean length of 1-1.2kb, terminate in a G-rich 3' overhang and appear to form T-loops (Figueiredo et al., 2002; Scherf et al., 2001; Bottius et al., 1998; Vernick and McCutchan, 1988; De Cian A. et al., 2008; Mancio-Silva et al., 2008; Figueiredo et al., 2005). *P. falciparum* chromosome ends are characterized by large blocks of HP1/H3K9me3-demarcated heterochromatin, which provides the framework for clonally variant expression of subtelomeric gene families primarily encoding polymorphic surface antigens (Brancucci and Bertschi et al., 2014, Chapter 2, this thesis; Flueck et al., 2009; Lopez-Rubio et al., 2009; Salcedo-Amaya et al., 2009; Rovira-Graells et al., 2012). This is particularly relevant during bloodstream infection where parasites undergo unlimited rounds of intra-erythrocytic mitotic proliferation (schizogony). The best-studied example is antigenic variation of the major virulence factor PfEMP1, encoded by the 60-member *var* gene family (Scherf et al., 2008; Guizetti and Scherf, 2013). *var* genes are silenced in a HP1-dependent manner and represent the most telomere-proximal genes on each chromosome (Perez-Toledo et al., 2009; Brancucci and Bertschi et al., 2014, Chapter 2, this thesis; Flueck et al., 2009; Gardner et al., 2002). A complex epigenetic mechanism of singular gene choice ensures that only a single *var* locus is activated and that *in situ* transcriptional switching between *var* loci, and hence antigenic variation of PfEMP1, can occur (Guizetti and Scherf, 2013; Voss et al., 2014). As a consequence, parasites can escape adaptive immune responses and establish chronic blood infection. This ultimately supports the continuous production of non-proliferative sexual forms (gametocytes) that are essential for human-to-human transmission via the mosquito vector (Baker, 2010).

The lack of known telomere-specific factors in *P. falciparum* has so far largely precluded the targeted investigation of essential telomere biology in this important parasite. Here, we describe the experimental *de novo* identification of the *P. falciparum* telomere repeat-binding factor PfTRF. Surprisingly we find that PfTRF binds ds telomere repeats via an array of C_2H_2-type zinc finger (ZnF) domains. This represents the first example of a C_2H_2-type TRF in eukaryotes, suggesting that *Plasmodium* parasites employ an evolutionary divergent molecular complex to preserve telomere function. *In vivo*, PfTRF binds to all 28 chromosome termini and, intriguingly, also to telomere repeat-like clusters in subtelomeric *var* gene promoters. We further show that PfTRF regulates telomere length homeostasis and is required for cell cycle progression and parasite proliferation.

4.3 Results

4.3.1 Identification of a sequence-specific telomere repeat-binding activity in *P. falciparum* nuclear extracts

In order to identify a potential TRF-like activity in *P. falciparum* we performed electromobility shift assays (EMSA) using a natural 42bp double-stranded (ds) telomere repeat sequence termed Tel6mer (Pf3D7_12_v3; position 475-516; PlasmoDB release 26). A scrambled sequence of identical length and base composition served as negative control (scrTel6mer). The Tel6mer probe indeed formed a sequence-specific DNA-protein complex when incubated with parasite nuclear extracts (Figure 1A). Complex formation occurred only with ds DNA and was completely dependent on the presence of Mg^{2+} and Zn^{2+} ions. We also established that salt concentrations above 500mM KCl prevented formation of the complex (Figure S1).

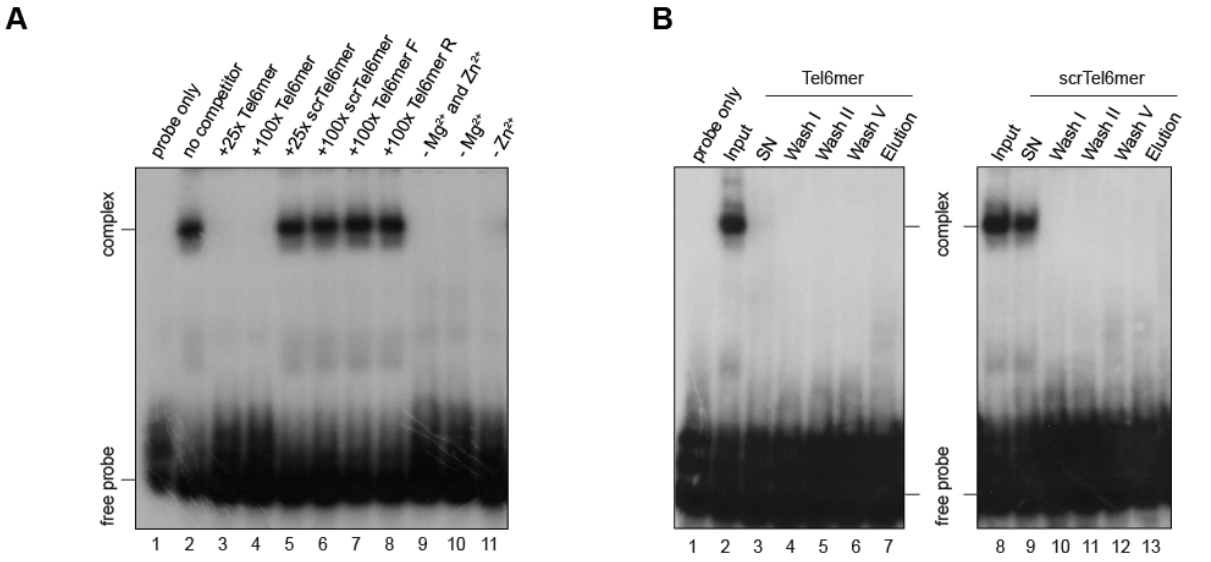

Figure 1. Identification and purification of a sequence-specific telomere-repeat binding activity in *P. falciparum*

(A) EMSA showing formation of a sequence-specific complex between ds Tel6mer DNA and an unknown activity in parasite nuclear extracts. Lanes 3-8: Fold molar excess of unlabelled competitor DNA is indicated above each lane. ss oligonucleotide competitors are denoted by F (forward) or R (reverse). Lanes 9-11: omission of Mg^{2+} and/or Zn^{2+} from the binding buffer is indicated. See also Figure S1. (B) Affinity purification was conducted by incubating nuclear extract with beads carrying the Tel6mer (left panel, lanes 2-7) or scrTel6mer sequence (right panel, lanes 8-13). Telomere repeat-binding activity was tested by EMSA in the input, supernatant, wash and elution fractions. SN, supernatant. See also Tables 1 and S1.

To identify this activity we purified nuclear proteins based on their affinity to telomere repeats using a biotinylated Tel6mer sequence immobilised on streptavidin agarose beads. As expected, the telomere repeat-binding activity was specifically depleted in the supernatant only after incubation with Tel6mer but not scrTel6mer DNA (Figure 1B). For unknown reasons, we repeatedly failed to observe this activity by EMSA in the 2M KCl elution and suspect this may have been due to protein instability. We nevertheless analysed the proteins eluted from both Tel6mer and negative control beads by liquid chromatography tandem mass spectrometry (LC-MS/MS). 108 and 118 parasite proteins represented by two or more unique tryptic peptides were detected in the Tel6mer and scrTel6mer eluates, respectively (Table S1). Importantly, we found eleven proteins exclusively in the Tel6mer elution and most of these carry domains implicated in protein-nucleic acid interactions (Table 1).

Gene ID PlasmoDB	Annotation	Domains	Domain ID	#
PF3D7_0605100	RNA-binding protein, putative	KH (4x)	pfam00013	13
PF3D7_1014100	conserved Plasmodium protein, unknown function	-	-	3
PF3D7_1326300	RNA-binding protein, putative	RRM	pfam00076	3
PF3D7_1468900	conserved Plasmodium protein, unknown function	DUF4187	pfam13821	3
PF3D7_0935800	cytoadherence linked asexual protein 9 (CLAG9)	CLAG	pfam03805	3
PF3D7_1209300	zinc finger transcription factor, putative (KROX1)	C_2H_2 ZnF (9x)	sd00017	2
PF3D7_0408300	zinc finger, RAN binding protein, putative	ZF RanBP	pfam00641	2
PF3D7_0422500	pre-mRNA-splicing helicase BRR2, putative (BRR2)	DEAD	pfam00270	2
PF3D7_0514200	conserved Plasmodium protein, unknown function	-	-	2
PF3D7_0812500	RNA-binding protein, putative	RRM	pfam00076	2
PF3D7_1027800	60S ribosomal protein L3 (RPL3)	ribosomal_L3	pfam00297	2

Table 1. Proteins exclusively detected in the Tel6mer elution

Putative domains were predicted using the Conserved Domain Database at NCBI (E-value <0.01) (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer et al., 2015). The number of unique peptides identified by LC-MS/MS is indicated in the last column (#) (see also Table S1).

The top-ranked protein (PF3D7_0605100) contains several KH domains, which bind nucleic acids and are involved in poly-C sequence recognition of telomere repeats in other organisms (Marsellach et al., 2006; Du et al., 2004). Strikingly, this list also featured a protein harbouring nine C-terminal C_2H_2-type zinc fingers (ZnF), annotated as putative zinc finger transcription factor KROX1 (PF3D7_1209300). This protein sparked our interest because (1) the binding of the *P. falciparum* telomere repeat-binding activity to telomere repeats is Zn^{2+}-dependent (see above); (2) it is the only protein in this list carrying known sequence-specific DNA-binding domains; and (3) a computational binding site predictor for C_2H_2-type ZnFs (zf.princeton.edu) (Persikov and Singh, 2014) predicted two G-rich telomere repeat-like DNA elements as putative targets sites for ZnFs 1-3 and 4-6, respectively (data not shown).

4.3.2 Experimental validation identifies a C_2H_2-type zinc finger protein as the *P. falciparum* telomere repeat-binding factor PfTRF

To verify if this C_2H_2 ZnF protein indeed binds to telomere repeats we conducted a series of *in vivo* and *in vitro* experiments. Indirect immunofluorescence assays (IFA) and live cell imaging revealed that an ectopically expressed GFP-tagged version of this factor localised to distinct spots at the nuclear periphery, adjacent to or overlapping with the chromosome end marker PfHP1 (Flueck et al., 2009) (Figures 2A and 2B). In contrast, a GFP-fusion of the KH domain protein (PF3D7_0605100) did not primarily localise to chromosome ends even though it displayed a punctate nuclear pattern and some association with PfHP1 (Figure S2). EMSAs using nuclear extracts from these parasites showed that the Tel6mer complex was super-shifted in presence of anti-GFP antibodies, demonstrating that the GFP-tagged C_2H_2 ZnF protein is indeed part of this complex (Figure 2C). This result was independently confirmed in pull-down experiments where the GFP-tagged C_2H_2 ZnF protein bound specifically to immobilised Tel6mer but not scrTel6mer DNA (Figure 2D) .

To unequivocally demonstrate that the C_2H_2 ZnF protein binds directly to telomere repeats it was necessary to observe this interaction using purified recombinant protein. All efforts to express in a suitable form either the full length protein or a fragment carrying all nine C_2H_2-type ZnFs in *E. coli* failed (data not shown). However, we achieved expression and purification of a recombinant soluble protein containing the first five annotated ZnFs ($5xC_2H_2$; amino acids 1023-1292), which are predicted to bind to G-rich DNA motifs (Figure S3). This purified recombinant polypeptide indeed bound to telomere repeat DNA in EMSA with similar sequence-specificity as compared to experiments using parasite nuclear extracts (Figure S3). Together, these data unambiguously identify the C_2H_2-type ZnF protein PF3D7_1209300 as the *P. falciparum* telomere repeat-binding factor, here dubbed PfTRF.

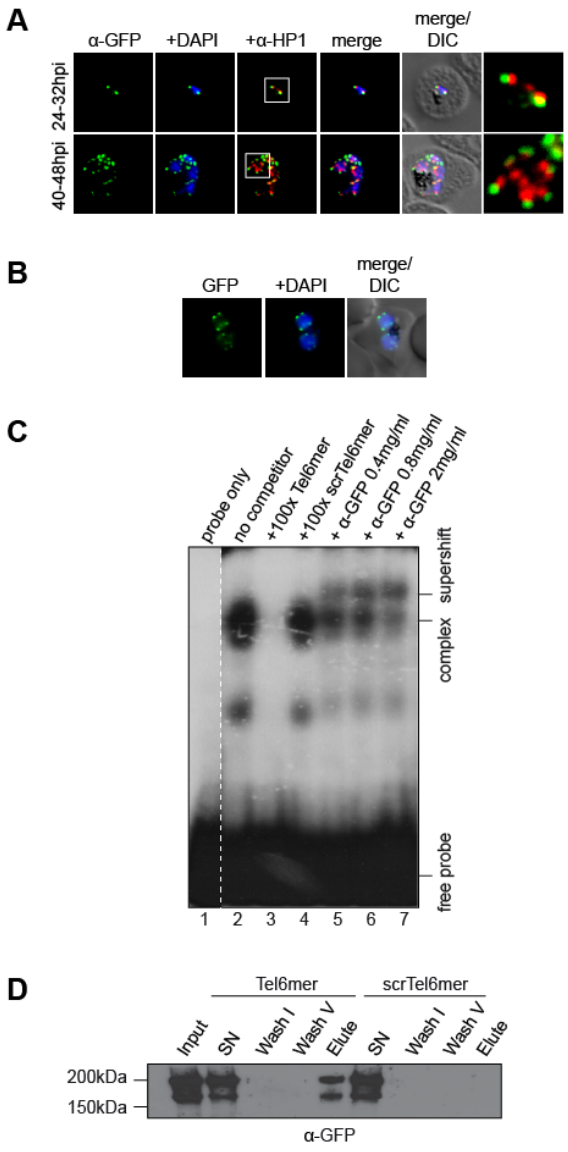

Figure 2. The C_2H_2 ZnF protein (PF3D7_1209300) localises to chromosome end clusters and associates with telomere repeats *in vitro*

(A) IFAs of parasites expressing an ectopic GFP-tagged version of the C_2H_2 ZnF protein. Protein localisation was visualised in trophozoites (24-32hpi) and multinucleated schizonts (40-48hpi) using anti-GFP (green) and anti-PfHP1 antibodies (red). Nuclei were stained with DAPI (blue). DIC, differential interference contrast. hpi, hours post-invasion. White frames refer to the magnified view in the rightmost images. See also Figure S2. (B) Live cell imaging of parasites expressing an ectopic GFP-tagged version of the C_2H_2 ZnF protein in trophozoites (24-32hpi). Nuclei were stained with DAPI (blue). (C) Super-shift EMSA using nuclear extract prepared from parasites expressing an ectopic GFP-tagged version of the C_2H_2 ZnF protein. Lanes 3-4: Fold molar excess of unlabelled competitor DNA is indicated above each lane. Lanes 5-7: Super-shift EMSA in presence of increasing amounts of anti-GFP antibodies. See also Figure S3. (D) Western blot analysis of *in vitro* pull-down fractions. Nuclear extract (input) from parasites expressing an ectopic GFP-tagged version of the C_2H_2 ZnF protein were incubated with either Tel6mer or scrTel6mer DNA immobilised on agarose beads. SN, supernatant.

4.3.3 ChIP-Seq reveals *in vivo* binding of PfTRF to telomeres, subtelomeric *var* gene promoters and 5S rDNA loci

To further characterise PfTRF *in vivo*, we generated transgenic parasites expressing either PfTRF-GFP (3D7/TRF^{GFP}) or PfTRF-3xHA (3D7/TRF^{HA}) from the endogenous locus (Figure S4). As expected, both PfTRF-GFP and PfTRF-3xHA localised to distinct foci at the nuclear periphery throughout the intra-erythrocytic cell cycle (Figures 3A-3C). The number of PfTRF fluorescence foci as well as total PfTRF expression levels (Figure S5) increased in schizonts, which is consistent with the consecutive rounds of genome duplication during schizogony. Furthermore, quantitative co-localisation analysis by confocal laser-scanning microscopy confirmed the exclusive co-localisation of PfTRF with PfHP1 at chromosome-end clusters (Manders coefficient: 0.95+/-0.05) (Figure 3D).

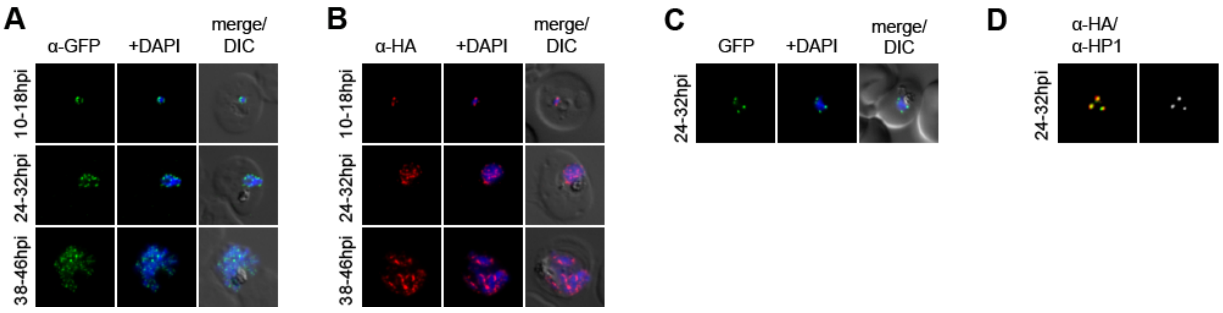

Figure 3. Localisation of endogenous PfTRF during the intra-erythrocytic cell cycle

(A) Localisation of endogenous PfTRF-GFP was visualised by IFA using anti-GFP antibodies (green). Nuclei were stained with DAPI (blue). DIC, differential interference contrast. See also Figures S4 and S5. (B) Localisation of endogenous PfTRF-HA was visualised by IFA using anti-HA antibodies (red). Nuclei were stained with DAPI (blue). DIC, differential interference contrast. See also Figures S4 and S5. (C) Live cell imaging of endogenous PfTRF-GFP (green). Nuclei were stained with DAPI. (D) Co-localisation of PfTRF and PfHP1 was visualised by confocal laser-scanning microscopy in 3D7/TRF^{HA} parasites using anti-HA (red) and anti-HP1 antibodies (green). Pearson co-localisation map of deconvolved images visualises overlapping PfTRF and PfHP1 signals (right image).

To map PfTRF binding sites throughout the genome at high resolution we performed chromatin immunoprecipitation (ChIP) followed by high-throughput sequencing (ChIP-Seq) for both transgenic parasite lines. Anti-GFP and anti-HA ChIP and corresponding input samples from each line were sequenced single-end for 75bp and locally enriched sites were identified using the peak calling algorithm MACS2 (Zhang et al., 2008) (www.github.com/taoliu/MACS/). As expected, PfTRF showed clear enrichment at the telomeric repeat tracts on all 14 chromosomes (Figure 4A). Interestingly, we also observed PfTRF enrichment in the promoters of all *ups*B-type *var* genes (23 subtelomeric and one chromosome-internal locus) (Lavstsen et al., 2003). These *ups*B-associated sites typically contained 6-8 TT(T/C)AGGG repeat motifs and occurred at a remarkably conserved distance upstream of the ATG start codon and downstream of the previously identified SPE2 motif arrays that are bound by the sequence-specific DNA-binding factor PfSIP2 (Flueck et al., 2010) (Figures 4B, 4C and S6). Competition EMSA demonstrated that PfTRF indeed binds to these telomere repeat-like *ups*B promoter elements (Figure S6). Besides this, seven additional intra-chromosomal sites of PfTRF enrichment were detected and called in the PfTRF-3xHA ChIP-Seq only (Figure 4D and S7). Interestingly, three of these peaks localised to the three adjacent 5S rDNA genes on chromosome 14 (PF3D7_1418500, PF3D7_1418600, PF3D7_1418700) (Figure 4D). This finding is notable because in other eukaryotes 5S rDNA transcription is regulated by the transcription factor TFIIIA, which carries nine C_2H_2-type ZnFs and binds with high specificity to a G-rich internal control region within the 5S rDNA gene body (Miller et al., 1985; Layat et al., 2013; Pieler et al., 1987).

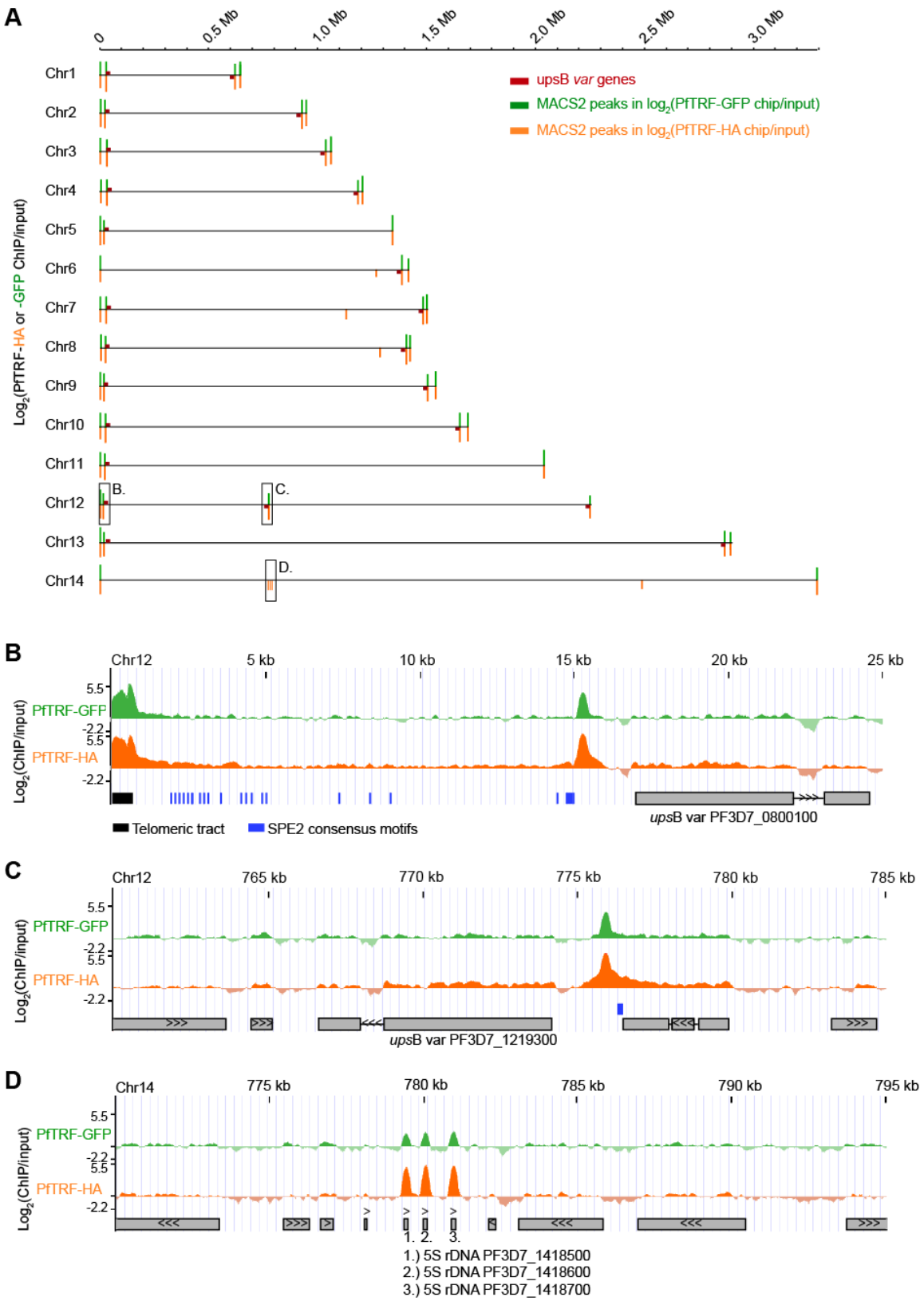

Figure 4. Genome-wide analysis of PfTRF binding sites by ChIP-Seq

(A) Genome-wide overview of the PfTRF enrichment sites as identified by ChIP-Seq in 3D7/TRF^{GFP} (green peaks) and 3D7/TRF^{HA} parasites (orange peaks). Position of *ups*B type *var* genes is indicated in red. Open boxes indicate the regions shown in panels B-D. (B-D) Screenshots of PfTRF ChIP-Seq data at three different genomic regions on chromosomes 12 and 14. Tracks depict normalised $\log_2$

ChIP over input ratios for PfTRF-GFP and PfTRF-3xHA, respectively. The telomeric tract and position of SPE2 consensus motifs are indicated. See also Figures S6 and S7.

4.3.4 PfTRF is required for cell cycle progression and telomere length homeostasis

Since the *pftrf* locus was refractory to deletion (data not shown) we created a conditional loss-of-function mutant (3D7/TRF$^{DD\text{-}ON}$) expressing endogenous PfTRF-GFP fused to a C-terminal FKBP destabilisation domain (DD) (Figure S4). This system allows for the rapid degradation of fusion proteins in absence of the stabilising compound Shield-1 (Armstrong and Goldberg, 2007; Banaszynski et al., 2006). PfTRF showed the expected perinuclear localisation in 3D7/TRF$^{DD\text{-}ON}$ parasites, whereas in absence of Shield-1 (3D7/TRF$^{DD\text{-}OFF}$) PfTRF expression was barely detectable by IFA (Figure 5A). Similarly, Western blot analysis revealed that PfTRF expression was already lower in 3D7/TRF$^{DD\text{-}ON}$ parasites compared to the control, and was even further reduced in 3D7/TRF$^{DD\text{-}OFF}$ parasites where only residual protein levels were detectable (Figure 5B). Interestingly, 3D7/TRF$^{DD\text{-}ON}$ parasites took slightly longer to complete their intra-erythrocytic cell cycle compared to the 3D7/TRF^{GFP} control, and this phenotype was even more pronounced in 3D7/TRF$^{DD\text{-}OFF}$ parasites and gradually increased the longer the population was propagated in absence of Shield-1 (data not shown). In addition, the parasite multiplication rate (PMR) was almost two-fold lower in 3D7/TRF$^{DD\text{-}OFF}$ (3.2-fold +/-0.7 SD) compared to 3D7/TRF^{GFP} control populations (5.75-fold +/-0.6 SD).

To study this interesting phenotype in more detail, we performed single cell DNA content analysis in 3D7/TRF$^{DD\text{-}OFF}$ parasites cultured in absence of Shield-1 for eight weeks (20 generations) and in 3D7/TRF^{GFP} control parasites (Figure 5C).This experiment showed that the 3D7/TRF$^{DD\text{-}OFF}$ population requires approximately eight additional hours to complete schizogony, and that individual 3D7/TRF$^{DD\text{-}OFF}$ parasites display substantial heterogeneity in cell cycle length, resulting in a rapid loss of synchronous cell cycle progression. Moreover, these results also confirmed the reduced PMR of 3D7/TRF$^{DD\text{-}OFF}$ parasites compared to the control population.

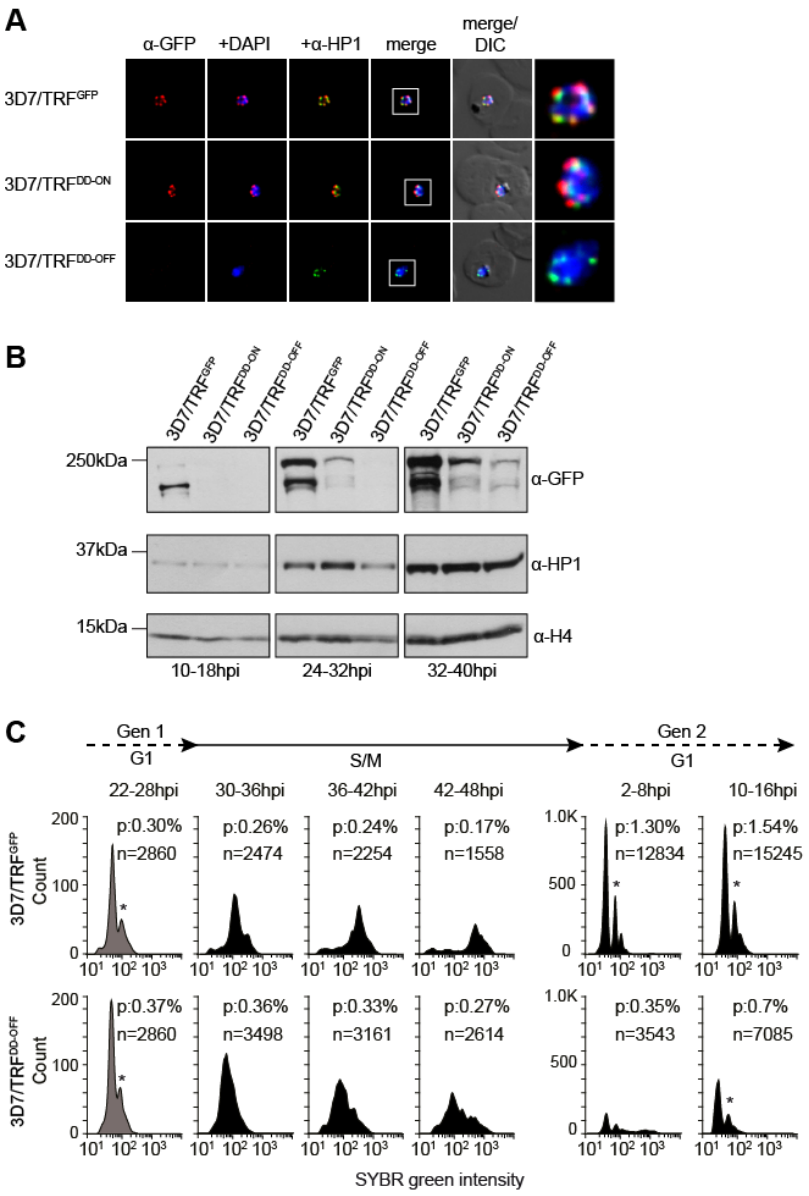

Figure 5. PfTRF is required for efficient mitotic cell cycle progression and parasite multiplication

(A) IFAs show correct localisation of PfTRF-GFP-DD in 3D7/TRF$^{DD\text{-}ON}$ parasites and lack of expression in 3D7/TRF$^{DD\text{-}OFF}$ parasites. Images were taken at trophozoite stage (24-32hpi). PfTRF-GFP-(DD) and PfHP1 were visualised using anti-GFP (green) and anti-PfHP1 antibodies (red), respectively. Nuclei were stained with DAPI (blue). DIC, differential interference contrast. White frames refer to the magnified view in the rightmost images. See also Figure S4. (B) Western blot analysis of whole cell lysates of 3D7/TRF^{GFP}, 3D7/TRF$^{DD\text{-}ON}$ and 3D7/TRF$^{DD\text{-}OFF}$ parasites harvested at three TPs during the intra-erythrocytic cell cycle. For each lane the amount of extract loaded corresponds to equal parasite numbers. Membranes were probed with anti-GFP antibodies (the lower band represents a consistently observed degradation product). Anti-PfHP1 and anti-H4 antibodies were used as controls. See also Figure S4. (C) Cell cycle progression was monitored in highly synchronous 3D7/TRF^{GFP} (top series) and 3D7/TRF$^{DD\text{-}OFF}$ populations (bottom series) by flow cytometry. The assay was started in late G1 phase (1N; 22-28hpi) and five subsequent TPs were analysed after 8, 14, 20, 28 and 36 hours to capture completion of schizogony (30-48hpi) and parasite re-invasion (2-16hpi, second generation). The “hpi” windows refer to the cell cycle progression

observed for the control parasites (confirmed by inspection of Giemsa-stained blood smears). hpi, hours post-invasion. n, number of gated iRBCs. p, parasitemia. asterisk, double infections.

We next asked if PfTRF also controls telomere length homeostasis. To this end, we performed Southern blot analysis of digested genomic DNA from 3D7/TRF$^{DD\text{-}OFF}$ parasites cultured in absence of Shield-1 for eight weeks, two weeks or three days. 3D7/TRF$^{DD\text{-}ON}$ and two control lines (3D7/TRF^{GFP} and parental 3D7 wild type) were cultured for eight weeks in parallel. Consistent with the average telomere length in *P. falciparum* (Figueiredo et al., 2002), the controls showed a mean telomere length of 1-1.2kb (Figure 6). Telomeres in 3D7/TRF$^{DD\text{-}ON}$ parasites were elongated to a mean length of approximately 1.8kb. Most strikingly, 3D7/TRF$^{DD\text{-}OFF}$ parasites acquired progressively longer telomeres reaching a mean size of up to 3kb after eight weeks of culture in absence of Shield-1, showing that PfTRF acts as a negative regulator of telomere length.

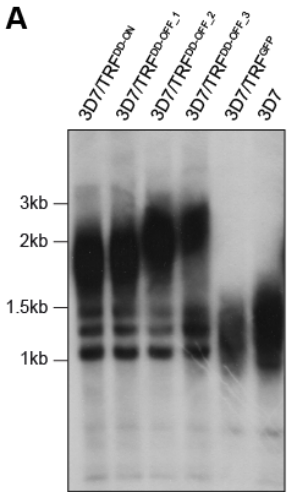

Figure 6. PfTRF regulates telomere length homeostasis

Telomere length analysis in 3D7/TRF$^{DD\text{-}ON}$ parasites and in 3D7/TRF$^{DD\text{-}OFF}$ parasites cultured in absence of Shield-1 for 3 days (TRF^{OFF_1}), 2 weeks (TRF^{OFF_2}) and 8 weeks (TRF^{OFF_3}). Genomic DNA was digested with the restriction enzymes *Alu*I, *Dde*I, *Mbo*II and *Rsa*I. Mean telomere length was determined by Southern blot analysis using a telomere-specific probe. See also Figure S4.

4.4 Discussion

In this study, we report the experimental *de novo* identification and functional characterisation of the telomere repeat-binding factor PfTRF in the malaria parasite *P. falciparum*. To the best of our knowledge, PfTRF is the first example of a telomere repeat-binding factor in eukaryotes that binds to duplex telomere repeats via C_2H_2-type ZnFs rather than a MYB domain. Still, the functional properties of PfTRF are strikingly similar to those described for MYB-type TRFs. We show that PfTRF (i) binds with high specificity to duplex telomere repeat arrays both *in vitro* and *in vivo*; (2) regulates telomere length homeostasis; and (3) is required for efficient cell cycle progression and proliferation. Our findings therefore provide compelling evidence that PfTRF represents a functional analog of MYB-type TRFs and allow us to speculate about a novel route of convergent evolution of TRFs in eukaryotes.

MYB-type TRFs (homologs of HsTRF1/TRF2 and/or ScRAP1) have been described in representatives of four of the five eukaryotic supergroups (Opisthokonts, Amaebazoa, Archeaplastida, Excavates) (Horvath, 2000; Giraud-Panis et al., 2010). In light of this wide conservation it has been proposed that TRFs evolved from a few ancestral MYB-type transcriptional regulators and were subsequently preserved during evolution by strong positive selection (Horvath, 2000; Rhodes et al., 2002).

However, functional homologs of MYB-type TRFs have not been identified in the SAR supergroup, which includes the Alveolate phylum that also comprises *P. falciparum* as well as *Tetrahymena thermophila*, a model organism for the study of telomere biology. This raises the question as to whether these organisms do not rely on TRFs for telomere protection, or if divergent/unrelated factors fulfil this task. Our discovery of PfTRF as the first duplex telomere repeat-binding factor in Alveolates favours the latter scenario and suggests that TRFs evolved independently of MYB-type TRFs from an ancestral C_2H_2-type ZnF protein. This factor might be the ancestor of current day TFIIIA, a conserved basal transcription factor of eukaryotes. In all eukaryotes studied so far, TFIIIA is encoded by a single gene and required exclusively to drive the RNA PolIII-dependent transcription of 5S rDNA loci (Layat et al., 2013). TFIIIA contains nine C_2H_2-type ZnFs, of which the first three are essential for the binding of TFIIIA to the C Box, a complex G-rich control element in the internal promoter of 5S rDNA genes (Pieler et al., 1987; Engelke et al., 1980; Layat et al., 2013). PfTRF displays strikingly similar features with TFIIIA proteins. First, the C-terminal domain of PfTRF also contains nine C_2H_2-type ZnFs and bears considerable homology to TFIIIA (Aravind et al., 2003). Second, PfTRF binds to G-rich elements and the first five ZnFs are important for this interaction. Third, and most compelling, PfTRF binds *in vivo* to all three 5S rDNA loci present in the *P. falciparum* genome. Based on these grounds we hypothesise that PfTRF and TFIIIA evolved from a common ancestor, and it will be interesting to test if PfTRF still regulates 5S rDNA transcription. If the role of PfTRF in telomere protection represents the original function of TFIIIA that was subsequently lost in other eukaryotes, or if TFIIIA gained this function specifically during evolution of the Alveolate lineage remains to be investigated by phylogenetic and experimental approaches.

In this context it is worth mentioning that *P. falciparum* encodes a possible rudimentary homolog of MYB-type TRFs (PF3D7_0924800). This protein is much smaller and lacks the essential protein dimerization and interaction domains of MYB-type TRFs (Xin et al., 2008), is highly upregulated in differentiating sexual rather than proliferating asexual parasites (www.plasmodb.org), and an ectopically expressed version does not localise to chromosome ends *in vivo* (own observation). This observation may suggest that MYB-type TRFs evolved very early in the evolution of eukaryotes but were replaced by a TFIIIA-type TRF in all/certain lineages of the SAR supergroup.

Our functional data identified clear roles for PfTRF in telomere maintenance. Using ChIP-Seq, we show that PfTRF is specifically enriched at the telomeric tract on all 28 chromosome ends. Conditional depletion of PfTRF resulted in a dramatic increase in telomere length, showing that PfTRF is strictly required for telomere length homeostasis in proliferating blood stage parasites. In analogy to MYB-type TRFs, we expect PfTRF fulfils this task by acting as a negative regulator of telomerase, which is constantly active and essential in these life cycle stages (Bottius et al., 1998; Figueiredo et al., 2005; Religa et al., 2014).

PfTRF-depleted parasites also exhibited a strong defect in mitotic cell cycle progression, which in *P. falciparum* involves iterative rounds of genome duplication and nuclear division (schizogony). Our flow cytometry data suggest that depletion of PfTRF triggers cell cycle arrest/death in a subset of schizonts and/or causes a reduction in the average number of viable merozoites produced per schizont. Both of these scenarios could explain the impaired proliferation rate observed for the PfTRF-depleted population. While the underlying mechanisms remain unclear at this stage, this phenotype is fully consistent with the important role for TRFs in telomere protection and replication. In yeasts and mammals, de-protection of telomeres through depletion of TRFs or other shelterin components activates DNA damage response pathways, which ultimately elicit cell cycle arrest/death, and triggers DNA repair mechanisms of the non-homologous end joining (NHEJ) or homology-directed repair pathways (Karlseder, 2003; Palm and de Lange T., 2008; Giraud-Panis et al., 2010; O'Sullivan and Karlseder, 2010). Moreover, HsTRF1 and SpTaz1 are essential for terminal DNA replication by preventing replication fork stalling and HsTRF1 has also been implicated in telomere cohesion (Miller et al., 2006; Sfeir et al., 2009; Canudas et al., 2007; Giraud-Panis et al., 2010; Martinez and Blasco, 2015). In consideration of these findings, we speculate that the cell cycle defect in PfTRF-depleted parasites may also be related to the activation of DNA repair mechanisms (excluding the NHEJ pathway, which is absent in malaria parasites (Lee et al., 2014)) and/or impaired DNA replication at chromosome ends.

Intriguingly, next to the telomeric tracts we discovered a second set of PfTRF binding hotspots in subtelomeric heterochromatin, directly upstream of all the 24 *ups*B-type *var* genes encoded in the genome. A multiple alignment of these PfTRF-enriched sequences uncovered a highly conserved stretch of 6-8 consecutive telomere repeat-like motifs approx. 1700 bp upstream of each *ups*B *var* coding sequence. These elements are reminiscent of short interstitial telomere repeat sequences (ITSs), which have been described in many different eukaryotes (Ruiz-Herrera et al., 2008). Human TRF1, TRF2 and RAP1 indeed bind to a subset of ITSs in subtelomeric and genic regions and these interactions have been linked to regulating target gene expression (Yang et al., 2011; Simonet et al., 2011). Recently, it has also been shown that human telomeres loop back by interacting with ITSs in a TRF2-dependent manner, forming so-called interstitial t-loop structures that promote gene

silencing (Wood et al., 2014; Robin et al., 2014). In *S. pombe*, Taz1 regulates proper timing of late replication origins by binding to proximal ITSs (Tazumi et al., 2012). Although the functional consequences of the binding of PfTRF to ITS elements upstream of *ups*B *var* genes is unknown at this stage, it is notable that such extra-telomeric PfTRF binding sites were not found elsewhere in the parasite genome.

It is furthermore remarkable that these sites are always positioned 300bp downstream of the previously mapped SPE2 repeat arrays, which are bound by the ApiAP2 DNA-binding protein PfSIP2 (Flueck et al., 2010). Similar to PfTRF, PfSIP2 has a very exclusive genome-wide binding pattern restricted to *ups*B *var* loci and subtelomeric repeat regions. The exact function of PfSIP2 is still unknown but possible roles in *var* gene silencing, heterochromatin formation and DNA replication have been suggested (Flueck et al., 2010). In light of these peculiar associations, we anticipate that PfTRF plays a crucial role in heterochromatin formation and epigenetic regulation of mutually exclusive *var* gene expression. PfTRF may directly or indirectly recruit components of the silencing machinery such as SIR2, the H3K9-specific histone methyltransferase PfSET3, PfHP1, PfHDA2 and PfORC1, all of which are specifically enriched in telomeric/subtelomeric heterochromatin in *P. falciparum* (Hernandez-Rivas et al., 2013; Coleman et al., 2014; Flueck et al., 2009). Such a proposed function seems indeed likely, given that TRFs, RAP1 and other shelterin components in yeasts and humans are directly involved in the establishment of telomeric/subtelomeric heterochromatin (Blasco, 2007; Benetti et al., 2008; Kanoh et al., 2005; Moretti et al., 1994).

In conclusion, the results of our study constitute an important advance for our understanding of telomere biology and virulence gene expression in *P. falciparum* and the evolution of telomere maintenance mechanisms in eukaryotes. The identification of PfTRF as the first C_2H_2-type telomere repeat-binding factor suggests that malaria parasite employ an evolutionary divergent “shelterin-like” complex to maintain telomere integrity. The identification of PfTRF as the first known telomere-specific factor in *P. falciparum* will facilitate targeted approaches to investigate the molecular mechanisms involved in telomere maintenance and heterochromatin formation in malaria parasites in much greater detail. It will be particularly exciting to dissect the function of the large N-terminal region of PfTRF, which we expect to carry protein interaction modules to assemble the telomere protection complex and other factors involved in DNA repair, replication and heterochromatin formation. We are hopeful that further work in this field will deliver important new insight into essential mechanisms of genome maintenance and epigenetic control of gene expression in these important pathogens.

4.5 Experimental Procedures

4.5.1 Parasite Culture and Transfection

P. falciparum culture and transfection was performed as described (Trager and Jensen, 1978; Voss et al., 2006). Growth synchronization was achieved by repeated sorbitol treatments (Lambros and Vanderberg, 1979). Transgenic cell lines are described in Supplemental Experimental Procedures and were grown in the presence of 4nM WR99210 (WR) (and 625nM Shield-1 for 3D7/TRF-GFP$^{DD\text{-}ON}$).

4.5.2 Preparation of parasite nuclear extracts

Nuclei were isolated at 10-18hpi as described previously (Voss et al., 2003) with minor modifications. Nuclei were resuspended in one pellet volume of high salt extraction buffer (20mM Hepes pH7.9; 800mM KCl, 1mM EDTA, 2mM DTT, 1x protease inhibitor (Roche, complete EDTA-free # 11873580001), 10% Glycerol; 1%Triton X-100) and proteins extracted by gentle vortexing at 4°C for 30min. After centrifugation at 8000g for 5min, the supernatant was stored at -80°C.

4.5.3 Electromobility shift assay

EMSAs were carried out in a 20µl volume. 4µl crude nuclear extract was incubated in 1x EMSA buffer (20mM Hepes pH7.9, 60mM KCl, 2mM $MgCl_2$, 0.025mM $ZnCl_2$, 0.5mM EDTA, 0.1% Trition X-100, 10% Glycerol, 2mM DTT, 1x protease inhibitor) in presence of 750ng poly-dAdT and 200fmol 30b ss oligonucleotides as non-specific competitors. For competition experiments a 5- to 100-fold molar excess of specific ss/ds competitor was added, and anti-GFP antibodies (Roche diagnostics, #11814460001) were added for super-shift experiments. After incubation for 10min on ice, 20fmol of radiolabelled probe was added and samples were further incubated for 15min at RT prior to electrophoresis (15V/cm for 2h; 4°C) in 6% polyacrylamide gels (0.5xTBE). EMSAs using purified recombinant protein were performed as above using 2pmol Tel6mer probe and 0.2µl of purified recombinant protein (1.6 mg/ml). ds oligonucleotides were generated by annealing equimolar amounts of complementary sequences in 4xSSPE at 95°C followed by slow cooling to room temperature. Oligonucleotide sequences are listed in Table S2.

4.5.4 Affinity purification and capillary liquid chromatography tandem mass spectrometry (LC-MS/MS)

Affinity purification of proteins binding to telomere repeats was carried out under EMSA conditions using biotinylated DNA immobilised on streptavidin-coated agaraose beads

according to a previously described protocol (Flueck et al., 2010). Bound proteins were eluted with 2M KCl, precipitated with 10% TCA and analysed by LC-MS/MS as described (Flueck et al., 2010; Josling et al., 2015). Detailed protocols are provided in the Supplemental Experimental Procedures section.

4.5.5 Fluorescence microscopy

Live-cell fluorescence microscopy and IFAs were performed as described (Witmer et al., 2012; Brancucci and Bertschi et al., 2014). Confocal microscopy was carried out using a Zeiss LSM 700 confocal microscope (Carl Zeiss GmbH, Jena, Germany), with a 63x oil-immersion lens (1.4 numerical aperture). Subsequent deconvolution and co-localisation analysis was carried out using Huygene Remote manager (http://hrm.svi.nl; v3.2.2). Details regarding antibodies and image acquisition are provided in Supplemental Experimental Procedures.

4.5.6 Western Blot

Parasites were released from iRBCs by saponin lysis, resuspended in 8M Urea, 5%SDS, 40mM Tris pH6.8 and separated on NuPage 4-12% Bis-Tris gels (Novex). Detection of PfTRF-GFP(-DD), PfTRF-3xHA, PfHP1 and PfH4 was performed using mouse anti-GFP (Roche Diagnostics, #11814460001), 1:500; rat anti-HA 3F10 (Roche Diagnostics, #12158167001), 1:10'000; rabbit anti-HP1 (Brancucci and Bertschi et al., 2014) 1:5'000; rabbit anti-H4 (Abcam ab10158) 1:10'000.

4.5.7 Protein pull-down

Nuclear extracts from 3D7/TRF^{GFP} parasites were incubated with streptavidin agarose beads carrying either biotinylated Tel6mer or scrTel6mer DNA in 1xEMSA buffer. The incubation was supplemented with 750ng poly-dAdT and 200fmol random 30b ss oligonucleotide as non-specific competitor DNA. The supernatants were saved, beads were washed five times and proteins were eluted with 2%SDS.

4.5.8 Recombinant protein expression and purification

For recombinant expression in *E. coli* and subsequent purification, the first five C_2H_2 ZnF domains of PF3D7_1209300 were fused to a N-terminal maltose-binding protein (MBP) tag and a C-terminal 6xHis tag. The detailed procedure describing cloning, expression and purification is described in Supplemental Experimental Procedures.

4.5.9 ChIP-Seq

ChIPs were performed on 500ng formaldehyde-crosslinked, sonicated chromatin using either 1µg mouse anti-GFP (Roche Diagnostics, #11814460001) or 1µg rat anti-HA 3F10 (Roche Diagnostics, #12158167001). Decrosslinked eluates and input samples were used for library preparation using an optimised KAPA protocol (Kensche et al., *in revision at NAR*) followed by sequencing on a NextSeq500 system (Illumina). A detailed protocol for the entire workflow as well as for data analysis, processing and visualisation is provided in Supplemental Experimental Procedures.

4.5.10 Flow Cytometry

Parasites were tightly synchronized 18hrs apart to obtain a 6-hour growth window. DNA content analysis was carried out on six consecutive time points (generation 1: 22-28hpi, 30-36hpi, 36-42hpi, 42-48hpi; generation 2: 2-8hpi, 10-16hpi). 25µl packed RBCs were fixed in 4% formaldehyde/0.015% glutaraldehyde for 30min, washed three times in PBS and incubated in 300µl SYBR green staining solution (Sigma-Aldrich #S9430, diluted 1:5000 in PBS) for 20min. Cells were washed three times in PBS prior to flow cytometry analysis using a BD FACSCalibur instrument (BD Biosciences). One million events (RBCs) were measured (excitation 488nm; emission detection FL1 530nm ± 30nm) and a threshold of 10 on FL1 (SYBR green intensity) was applied to gate on the iRBC population. Acquired data was processed using FlowJo software (Version 10.0.5).

4.5.11 Southern blot

Telomere length analysis was performed as described (Figueiredo et al., 2002) with minor modifications. gDNA was digested with four restriction enzymes (*Alu*I, *Dde*I, *Mbo*II and *Rsa*I), separated in 0.5x TBE-buffered 0.8% agarose gel and transferred to a Hybond N+ membrane (Amersham). The blot was hybridised at 60°C with a ^{32}P-dATP-labelled Tel6mer probe, washed twice for 20min in 2xSSPE 0.1%SDS, and once for 10min in 0.2xSSPE 0.1%SDS, at 60°C.

4.6 Authors contributions

N.L.B. designed and performed experiments, analysed data, prepared illustrations and wrote the paper. C.G.T. performed and analysed ChIP-Seq experiments. I.N. expressed and purified recombinant proteins. R.H. generated the 3D7/TRF^{HA} parasite line. S.M. performed LC-MS/MS experiments. P.J. provided conceptual advice. P.J., R.B and T.S.V. provided resources. R.B. designed and supervised experiments and interpreted data. T.S.V. con-

ceived the study and designed, supervised, and analysed experiments and wrote the paper. All authors contributed to editing of the manuscript.

4.7 Acknowledgments

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4.9 Supplemental Information

Document S1: Figures S1-S7, Table S2, Supplemental Experimental Procedures and Supplemental References. Table S1: Proteins and peptides identified by Mascot and Sequest HT searches of the results obtained from LC-MS/MS analysis of nuclear proteins affinity-purified using immobilised Tel6mer DNA or scrTel6mer DNA (negative control). Please note that Table S1 is not inserted into the thesis, due to size. On request, it is available in the Gene Regulation Laboratory (Till S. Voss, Swiss TPH).

4.10 Supplemental Data

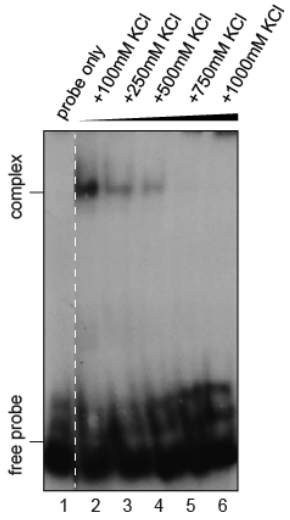


Figure S1. Gel-shift assay shows that elevated salt concentrations disrupt the telomere repeat-protein complex (related to Figure 1)

EMSA using ds Tel6mer probe shows complex formation when incubated with nuclear protein extracts. Increased salt concentrations prevent complex formation. Lane 1: free Tel6mer probe. Lanes 2-6: parasite nuclear extract. KCl concentrations in the binding buffer are indicated for each lane separately.

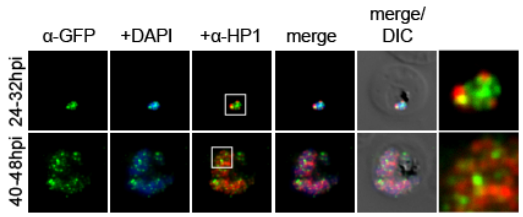


Figure S2. Localisation of the KH domain protein (PF3D7_0605100) by IFA (related to Figure 2)

IFAs of parasites expressing an ectopic GFP-tagged version of the KH domain protein PF3D7_0605100. Protein localisation was visualised in trophozoites (24-32hpi) and multinucleated schizonts (40-48hpi) using anti-GFP (green) and anti-PfHP1 (red) antibodies. Nuclei were stained with DAPI (blue). DIC; differential interference contrast. hpi, hours post-invasion. White frames refer to the magnified view in the rightmost images.

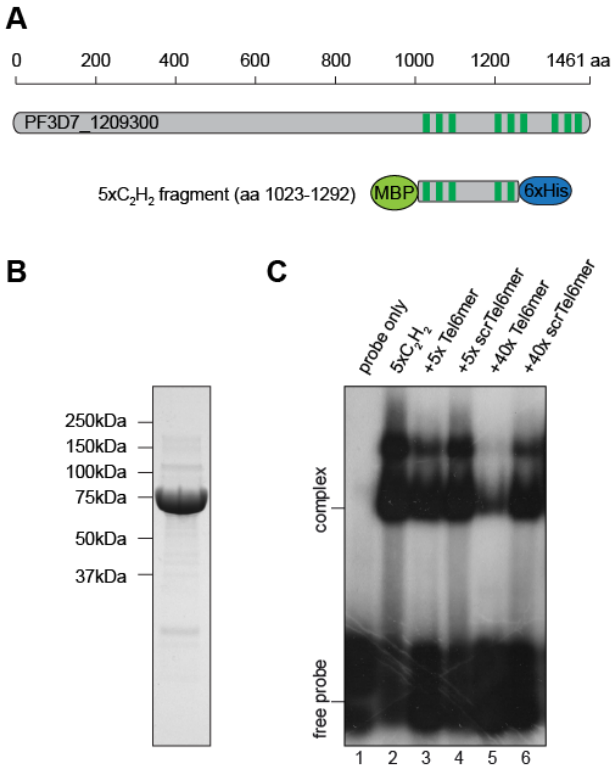


Figure S3. The C_2H_2 ZnF protein PF3D7_1209300 interacts with telomere repeats *in vitro* (related to Figure 2)

(A) Schematic map of the full length C_2H_2 ZnF protein encoded by PF3D7_1209300 (170kDa) (www.plasmodb.org) and the recombinant $5xC_2H_2$ fragment fused to a N-terminal maltose-binding protein (MBP) and C-terminal 6xHis tag, expressed in and purified from *E. coli* (expected size 73kDa). Green boxes indicate annotated C_2H_2-type ZnFs.(B) Coomassie-stained gel of the purified $5xC_2H_2$ fragment after a three-step purification strategy based on affinity to nickel, dextrin and heparin (see Supplemental Experimental Procedures). (C) Tel6mer DNA forms a specific complex when incubated with purified recombinant $5xC_2H_2$ fragment. Complex formation is competed in a sequence-specific manner only with a molar excess of Tel6mer (lane 3 and 5) but not scrTel6mer DNA (lanes 4 and 6). Lane 1: free Tel6mer probe. Lanes 2-6: Tel6mer probe incubated with purified recombinant $5xC_2H_2$. Lanes 3-6: fold molar excess of unlabelled competitor DNA is indicated above each lane.

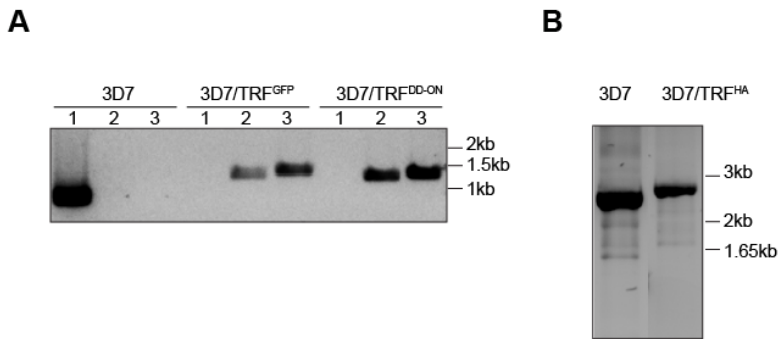


Figure S4. Verification of successful plasmid integration in 3D7/TRF^{GFP}, 3D7/TRF^{DD-ON} and 3D7/TRF^{HA} parasites (related to Figures 3-6 and Supplemental Experimental Procedures)

(A) PCR on gDNA isolated from 3D7 wild type, 3D7/TRF^{GFP} and 3D7/TRF^{DD-ON} parasites. Primer combinations are specific for diagnosing (1) the wild type *pftrf* locus (866 bp); (2) the successful integration of the targeting plasmids into the endogenous *pftrf* locus via single crossover recombination (1200 bp); and (3) the episomal *pftrf* targeting cassettes (1396 bp). (B) PCR on gDNA isolated from 3D7 wild type and 3D7/TRF^{HA} parasites. The primer pair amplifies the region surrounding the stop codon of the endogenous *pftrf* gene, yielding fragments of 2440 bp and 2584 bp in 3D7 wild type and 3D7/TRF^{HA} parasites, respectively. Primers are listed in Table S2.

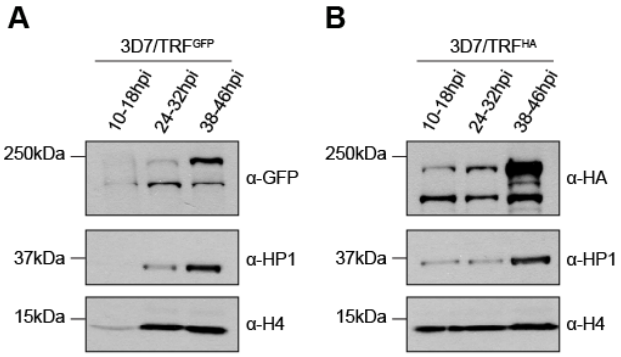
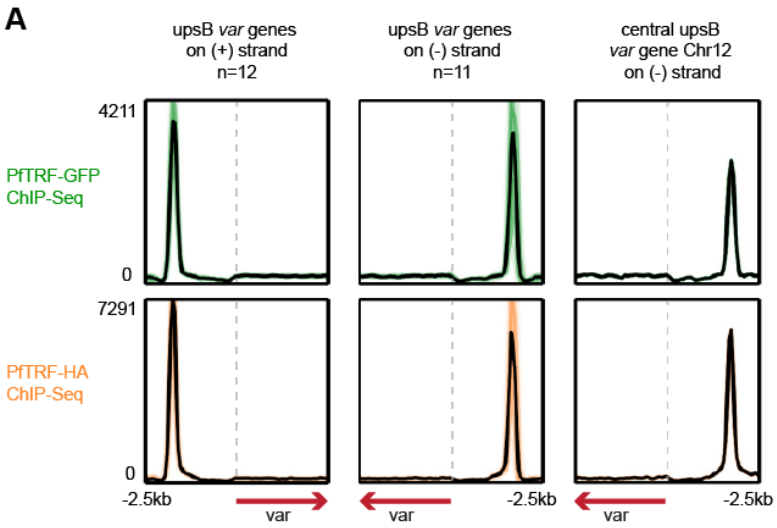


Figure S5. Expression of endogenous PfTRF during the intra-erythrocytic cell cycle (related to Figure 3)

Whole cell lysates were extracted at three consecutive time points (10-18hpi, 24-32hpi, 38-46hpi) from (A) 3D7/TRF^{GFP} parasites and (B) 3D7/TRF^{HA} parasites. For each TP the amount of protein lysate loaded corresponds to equal parasite numbers. In both cell lines PfTRF expression levels increased in schizonts. Western blots was probed with anti-GFP (3D7/TRF^{GFP}) and anti-HA antibodies (3D7/TRF^{HA}) (the lower bands represent consistently observed degradation products). Anti-PfHP1 and anti-H4 antibodies were used as controls.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upsB var genes on (-) strand n=11
central upsB var gene Chr12 on (-) strand
4211
PfTRF-GFP ChIP-Seq
0
7291
PfTRF-HA ChIP-Seq
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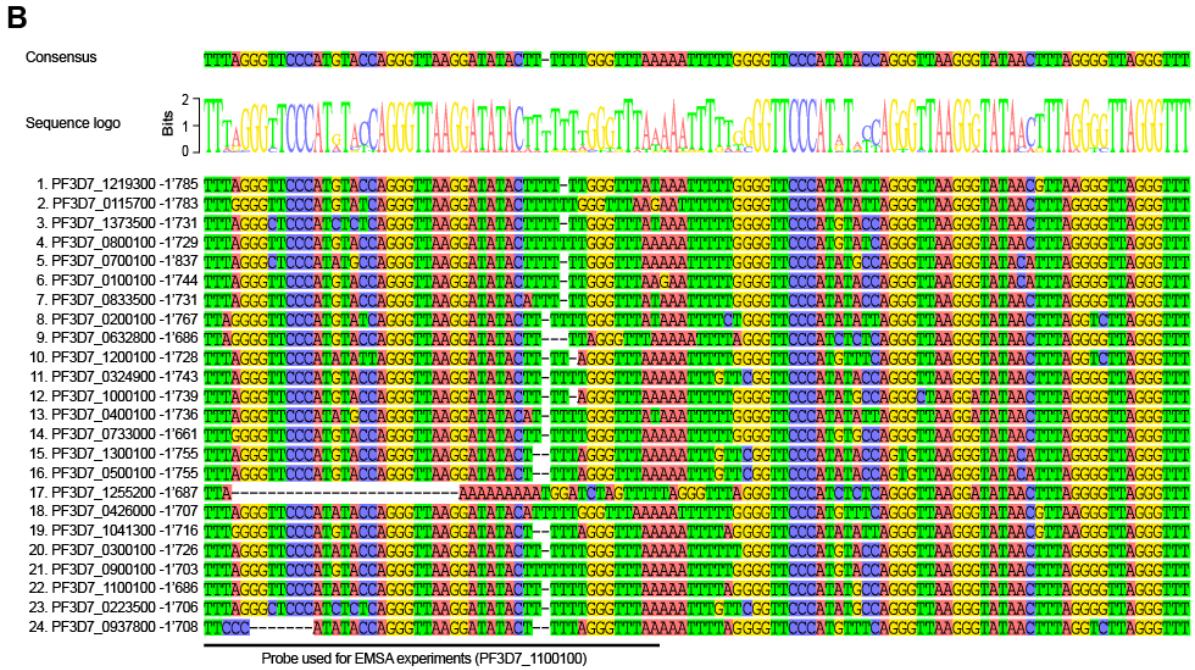
B
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3. PF3D7_1373500 -1'731
4. PF3D7_0800100 -1'729
5. PF3D7_0700100 -1'837
6. PF3D7_0100100 -1'744
7. PF3D7_0833500 -1'731
8. PF3D7_0200100 -1'767
9. PF3D7_0632800 -1'686
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18. PF3D7_0426000 -1'707
19. PF3D7_1041300 -1'716
20. PF3D7_0300100 -1'726
21. PF3D7_0900100 -1'703
22. PF3D7_1100100 -1'686
23. PF3D7_0223500 -1'706
24. PF3D7_0937800 -1'708
Probe used for EMSA experiments (PF3D7_1100100)

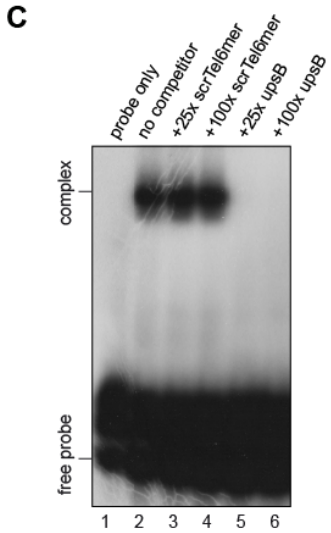
C
probe only
no competitor
+25x scrTel6mer
+100x scrTel6mer
+25x upsB
+100x upsB
complex
free probe
1
2
3
4
5
6

Figure S6. PfTRF binds to a conserved site in all *ups*B *var* gene promoters (related to Figure 4)

(A) Average PfTRF^{GFP} (green) and PfTRF^{HA} (orange) ChIP signals in a 5kb window around the ATG start codon (gray dashed line) of all 24 *ups*B *var* genes. The mean enrichment is shown as a black line while the 50th and 90th percentiles are coloured in dark and light colour, respectively. The panels represent all *ups*B *var* genes encoded on the sense strand (left panel) or antisense strand (middle panel). The single chromosome-central *ups*B *var* gene is shown in the right panel. (B) Multiple sequence alignment of PfTRF ChIP-Seq target regions in all 24 *ups*B-type *var* promoters. For each *ups*B *var* gene, the 2.5kb sequence directly upstream of the ATG start codon was retrieved from PlasmoDB (http://www.plasmoDB.org) and a multiple sequence alignment using the Clustal Omega sequence alignment tool was performed with default settings (http://www.ebi.ac.uk/Tools/msa/clustalo) (Sievers et al., 2011). The sequence alignment was visualised using Geneious Pro 5.1.7 software and a 108bp region centred on the ChIP-Seq PfTRF binding sites is shown. It reveals a high level of sequence similarity within these regions, which includes multiple telomere repeat-like G-rich elements. The sequence position within each *ups*B upstream sequence is indicated on the left (numbers refer to the position upstream of the *var* ATG start codon). The black bar on the bottom highlights the sequence used for competition experiments in EMSA (Figure S6C). (C) EMSA using a radiolabelled Tel6mer probe shows complex formation when incubated with parasite nuclear extract. Competition experiments with a 25-fold and 100-fold molar excess of a 50bp *ups*B promoter element (Pf3D7_1100100) containing telomere-like repeats prevents formation of complex. Lane 1: free Tel6mer probe. Lanes 2-6: parasite nuclear extract. Lanes 3-6: fold molar excess of unlabelled scrTel6mer or *ups*B competitor DNA is indicated above each lane.

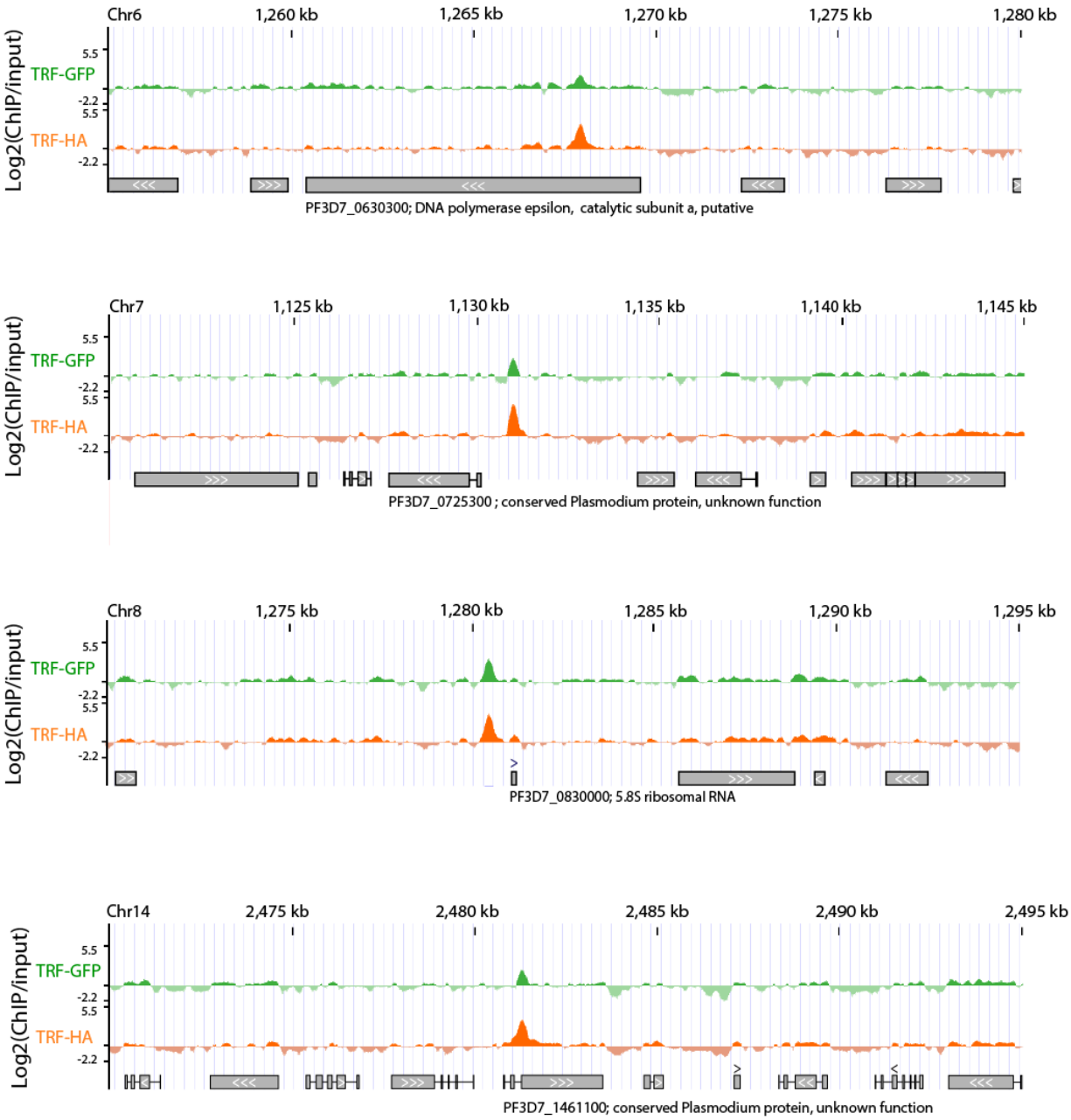

Figure S7. PfTRF enrichment at four chromosome-internal sites (related to Figure 4)

Screenshots of PfTRF ChIP-Seq data at four single chromosome-internal sites on chromosomes 6, 7, 8 and 14. Tracks depict the normalised $\log_2$ ChIP over input ratios for PfTRF-GFP and PfTRF-3xHA, respectively.

Table S1. Proteins and peptides identified by Mascot and Sequest HT searches of the results obtained from LC-MS/MS analysis of nuclear proteins affinity-purified using immobilised Tel6mer DNA or scrTel6mer DNA (negative control). (related to Figure 1 and Table 1)

Table not inserted into the thesis due to size. On request it is available in the Gene Regulation Laboratory (Till S. Voss, Swiss TPH).

Table S2. List of all oligonucleotide primers used in this study (related to Experimental Procedures and Supplemental Experimental Procedures)

Oligonucleotides for EMSA	**Sequence (5'-3')**
Tel6mer, fw	gatctttagggttcagggtttagggttcagggtttagggttcaggg
Tel6mer, rev	gatcccctgaaccctaaaccctgaaccctaaaccctgaaccctaaa
Scrambled, fw	gatccagatagtgcgatccatacgagtacagtctacgtgtccagtcatgc
Scrambled, rev	gcatgactggacacgtagactgtactcgtatggatcgcactatctggatc
upsB, fw	tttagggttcccatataccagggttaaggatatacttttttgggtttaaa
upsB, rev	tttaaacccaaaaaagtatatccttaaccctggtatatgggaaccctaaa

Oligonucleotides for affinity purification	**Sequence (5'-3')**
Tel6mer-biotin, fw	[Btn]-gcattttagggttcagggtttagggttcagggtttagggttcaggggatc
Tel6mer-biotin, rev	gatcccctgaaccctaaaccctgaaccctaaaccctgaaccctaaaatgc
Scrambled-biotin, fw	[Btn]-gcatgactggacacgtagactgtactcgtatggatcgcactatctggatc
Scrambled-biotin, rev	gatccagatagtgcgatccatacgagtacagtctacgtgtccagtcatgc

Primers for construct generation	**Sequence (5'-3')**	**Pf gene ID**
PF3D7_0605100_BamHI, fw	catgggatcctcaaaatgataaaacaacaaaaaagatcatatg	PF3D7_0605100
PF3D7_0605100 _NotI, rev	catggcggccgcaaaaccagtcgaatttaaaaattttgg	PF3D7_0605100
TRF_ pHcamGFP_Nhe, fw	catggctagcaaaaaatgagctgcatagattcaaaga	PF3D7_1209300
TRF _ pHcamGFP_NotI, rev	catggcggccgcatgagtgcatattaacgttgtgc	PF3D7_1209300
TRF_ pHGFP(DD)_PstI, fw	gtaggcggccatcgctgcagattacagaaggaaaagaaggtg	PF3D7_1209300
TRF _ pHGFP(DD)_NheI, rev	ggccgccatagctagctgagtgcatattaacgttgtg	PF3D7_1209300
MalE_K27_NdeI, fw	aaacatatgaaaatcgaagaaggtaaactg	
MalE_K27_BamHI, rev	tttggatccaccactactagtctgcgcgtctttcag	
TRF_I1023-E1292_BamHI, fw	ataaaaaaaggatccattaaaaaaaaaggtaag	PF3D7_1209300
TRF_I1023-E1292_XhoI, rev	gtttctcgagctcgttagttttattatgtgaac	PF3D7_1209300
TRF_HA,HomboxR_NheI, fw	aaacgtacgacacaaaaatgaatacgtcag	PF3D7_1209300
TRF_HA,HomboxR_NotI_rev	aaagcgcgcccgtagaaggaacacaag	PF3D7_1209300
TRF_HA,HomboxL_NheI, fw	aaagctagcaggacataaaataagtgaag	PF3D7_1209300
TRF_HA,HomboxL,BamHI rev	aaaggtacctgagtgcatattaacgttg	PF3D7_1209300

Primers for verification of integreation	**Sequence (5'-3')**	**Pf gene ID**
GFP_PCR 1_wt locus, fw	aaactacataacggtgtgatgg	PF3D7_1209300
GFP_PCR 1_wt locus, rev	tcattgttatacttttttctgacg	PF3D7_1209300
GFP_PCR 2_integrated, fw	aaactacataacggtgtgatgg	PF3D7_1209300
GFP_PCR 2_integrated, rev	ttgtgtgagttatagttgtattcc	
GFP_PCR 3_plasmid, fw	gggaaggtgttgctcaaatagtg	
GFP_PCR 3_plasmid, rev	ttgtgtgagttatagttgtattcc	
HA_PCR_integrated, fw	ggtaagaatgaaaatgatcgag	PF3D7_1209300
HA_PCR_integrated, rev	gtcaaatttgatggaggtcg	PF3D7_1209300

4.11 Supplemental Experimental Procedures

4.11.1 Transgenic cell lines

To generate transgenic cell lines expressing ectopic candidate proteins the full-length coding sequences were amplified from 3D7 gDNA. PCR products were cloned in frame with *gfp* into pH_camGFP (Witmer et al., 2012). To tag endogenous PfTRF with GFP or GFP-DD via single crossover homologous recombination, the 3' end of the *pftrf* coding sequence (780bp) was amplified from gDNA, and the *Pst*I/*Not*I-digested PCR fragments were cloned in frame with GFP and GFP-DD into derivatives of pH-GFP and pH-GFP-DD, respectively (Brancucci and Bertschi et al., 2014) (Birnbaum, Flemming & Spielmann, unpublished). To tag endogenous PfTRF with 3xHA, a double cross-over homologous recombination strategy was used (van Schaijk et al., 2010). A 1123 bp fragment corresponding to the 3' end of the *pftrf* gene and a 570 bp fragment corresponding to the *pftrf* 3' UTR were amplified from gDNA and used to replace the 5' and 3' homology arms, respectively, in pHHT-FRT-(GFP)-Pf5236 (van Schaijk et al., 2010). A sequence encoding the 3xHA tag was then inserted in frame with the *pftrf* coding sequence. After plasmid integration into the endogenous locus, the h*dhfr-gfp* selection cassette was removed by episomal expression of the enhanced FLP-recombinase as described (van Schaijk et al., 2010). This procedure left behind only a short sequence encoding the 3xHA tag and one FLIRT site appended in frame to the 3' end of the *pftrf* gene. Successful tagging of the endogenous *pftrf* locus in all lines was confirmed by PCR on gDNA (Figure S4). All primers used for cloning and PCR confirmation are listed in Table S2.

4.11.2 Fluorescence microscopy

IFAs were performed with methanol-fixed cells using the following primary antibodies: mouse anti-GFP (Roche Diagnostics, #11814460001) 1:100; rabbit anti-PfHP1 (Brancucci and Bertschi et al., 2014), 1:100; rat anti-HA 3F10 (Roche Diagnostics, #12158167001). Secondary antibodies: Alexa Fluor 568-conjugated anti-rabbit IgG (Molecular Probes; #A11011), 1:500; Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes; #A11001), 1:500; Alexa Fluor 488-conjugated anti-rat IgG (Molecular Probes; #A11006) 1:500. Images were taken at 96-fold magnification on a Leica DM 5000B microscope with a Leica DFC 345 FX camera, acquired via the Leica IM1000 software, and processed using Adobe Photoshop CS6. For each experiment, images were acquired and processed with identical settings.

4.11.3 Affinity purification of telomere repeat-binding proteins and capillary liquid chromatography tandem mass spectrometry (LC-MS/MS).

10^4pmol biotinylated ds Tel6mer or scrTel6mer DNA (Table S2) was immobilized on 200µl Pierce™ Streptavidin agarose beads (Thermo Scientific, #20347) according to the manufacturer's protocol. Beads were washed twice in 1xEMSA buffer and resuspended in 200µl 1xEMSA buffer. 600µl crude nuclear extract (corresponding to 750 ml parasite culture; 5% haematocrit; 8% parasitaemia) was diluted five times in 1xEMSA buffer and incubated for one hour at 4°C with 100 µl loaded agarose beads complemented with 40ng/µl poly-dAdT and 10fmol/µl random 30base ss oligonucleotide. The supernatants were saved and beads were washed five times in 1.3ml 1xEMSA buffer.

Proteins eluted from beads were precipitated with 10% TCA, washed twice in ice-cold acetone and air-dried. The protein pellets were dissolved in 10µl 100mM Tris-HCl, pH 8.0/6M Urea. After dissolving the pellet, urea was diluted by adding 20µl 100mM Tris-HCl, pH 8.0 and the sample was digested with 1µg endoproteinase LysC (Wako, Neuss, Germany) for 2hrs at 37°C followed by digestion with 1µg trypsin (Promega, WI, USA) for 18hrs at 37°C. The digest was acidified with TFA to 1% final concentration and the sample was desalted on a MicroSpin cartridge (The Nest Group, Southborough, MA, USA) according to the manufacturer's recommendations. The peptides were dried and analysed by capillary liquid chromatography tandem MS (LC/MS/MS) using a 300SB C-18 trap column (0.3x50mm) (Agilent Technologies, Basel, Switzerland) connected to a 0.1mm x 10cm capillary separation column packed with Magic C18 (5µm particle diameter). The capillary column was connected to an Orbitrap FT hybrid instrument (Thermo Finnigan, San Jose, CA, USA). A linear gradient from 2 to 60% solvent B (0.1% acetic acid and 80% acetonitrile in water) in solvent A (0.1% acetic acid and 2% acetonitrile in water) in 85min was delivered with a Rheos 2200 pump (Flux Instruments, Basel, Switzerland) at a flow of 50µl/min. A pre-column split was used to reduce the flow to approximately 500nl/min. 10µl sample was injected with an autosampler set to 4°C onto the trap column. The eluting peptides were ionized at 1.6kV. The mass spectrometer was operated in a data-dependent fashion so that peptide ions were automatically selected for fragmentation by collision-induced dissociation (MS/MS) in the Orbitrap. The MS/MS spectra were searched against a combined *P. falciparum* (www.plasmoDB.org; release 11.1)/human annotated protein database using Proteome Discoverer 1.4.0 (Thermo Scientific, Reinach, Switzerland) using the two search engines Mascot and SequestHT (Table S1). For the search, oxidized methionine, N-terminal protein acetylation and phosphorylation were used as variable modifications. The identifications were filtered to a false discovery rate of 1%.

4.11.4 Recombinant protein expression and purification

For recombinant expression in *E. coli* and subsequent purification, the first five ZnF domains of PF3D7_1209300 were fused to a N-terminal maltose-binding protein (MBP) tag and a C-terminal 6xHis-tag. The sequence encoding PfTRF amino-acids I1023-E1292 was amplified from gDNA using the primers listed in Table S2 and cloned into the *BamH*I/*Xho*I digested vector pETA_MBP. This plasmid was generated by replacing the peIB signal sequence of pET-20b(+) (Novagen) with a sequence encoding the MBP tag (K27-T392 of malE) followed by a short serine-glycine linker (SSG). For this purpose, a PCR product amplified from DH5α gDNA using the primers listed in Table S2 was ligated into the *Nde*I/*Bam*HI-digested pET-20b(+) vector.

The $5xC_2H_2$ fusion protein was expressed in Rosetta2 (DE3) cells (Novagen) at 25°C using auto-induction. The protein was purified under native conditions using the following three-step purification strategy: (1) Nickel-affinity purification: cells were resuspended in buffer Ni-A (50mM H3PO4, 0.5M NaCl, 20mM imidazole, 10mM 2-mercapto ethanol, pH 7.4) and lysed by sonication. The lysate was cleared by centrifugation prior to loading on a Ni^{2+}-charged HiTrap FF column (GE Healthcare). The column was washed with 15 column volumes (CV) buffer Ni-A and bound proteins were eluted with buffer Ni-E (50mM H_3PO_4, 0.5M NaCl, 225mM imidazole, 10mM 2-mercapto ethanol, NaOH to pH 7.4). (2) Dextrin-affinity purification: the eluate of step 1 was bound to an MBPtrap HP column (GE Healthcare). The column was washed with five CV buffer M (50mM HEPES-KOH, pH 7.5, 100mM NaCl, 0.5mM TCEP) and bound proteins were eluted with buffer H (buffer M supplemented with 10% glycerol, 0.15mM tri-sodium citrate, 0.1mM $ZnSO_4$ and 10mM maltose). (3) Heparin-affinity purification: the eluate of step 2 was bound to a HiTrap Heparin HP column (GE Healthcare) and proteins were eluted using a linear gradient (15 CV) from 0.1M to 1.5M NaCl in buffer H. The fractions containing the protein of interest were pooled and concentrated to 1.6mg/ml using an Amicon Ultra centrifugal filter (Millipore) with a 10k cut-off.

4.11.5 Chromatin Immunoprecipitations, high-throughput sequencing and data analysis

Synchronized 3D7/TRF^{GFP} and 3D7/TRF^{HA} schizonts were cross-linked by incubation with a final concentration of 1% formaldehyde for 15 min at 37ºC. The cross-linking reaction was quenched by addition of 0.125M glycine (final concentration). Nuclei were isolated by releasing parasites from iRBCs using 0.05% saponin followed by lysis in CLB (20mM Hepes, 10mM KCl, 1mM EDTA, 1mM EGTA, 0.65% NP-40, 1mM DTT supplemented with protease inhibitor). Nuclei were washed and snap-frozen in CLB supplemented with 50% glycerol. To obtain fragmented chromatin for ChIP, nuclei were resuspended in sonication buffer (50mM

Tris pH:8.0, 1% SDS, 10mM EDTA, supplemented with protease inhibitor) and sonicated for 20 cycles of 30 sec ON/30 sec OFF (setting high, Bioruptor^{TM} Next Gen, Diagenode). Fragment sizes ranged from 200-600bp as determined by decrosslinking a 50µl aliquot and running the purified DNA on a 1.5% agarose gel (data not shown). ChIPs were performed by incubating ~500 ng (DNA-content) sonicated chromatin from 3D7/TRF^{GFP} and 3D7/TRF^{HA} overnight while rotating at 4°C with 1µg mouse anti-GFP (Roche Diagnostics, #1181446 0001) or 1µg rat anti-HA 3F10 (Roche Diagnostics, #12158167001), respectively, in incubation buffer (5% Triton-X-100, 750mM NaCl, 5mM EDTA, 2.5mM EGTA, 100mM Hepes pH 7.6) with 10µl protA and 10µl protG Dynabeads (Life Technologies, 10008D and 10009D). Beads were washed for 5min at 4°C while rotating with 400µl of the following wash buffers: 2x wash buffer 1 (0.1% SDS, 0.1% DOC, 1% Triton-X100, 1mM EDTA, 0.5mM EGTA, 20mM Hepes pH 7.6), 1x wash buffer 2 (0.1% SDS, 0.1% DOC, 1% Triton-X100, 500mM NaCl, 1mM EDTA, 0.5mM EGTA, 20mM Hepes pH 7.6), 1x wash buffer 3 (250mM LiCl, 0.5% DOC, 0.5% NP-40, 1mM EDTA, 0.5mM EGTA, 20mM Hepes pH7.6), 2x wash buffer 4 (1mM EDTA, 0.5mM EGTA, 20mM Hepes pH7.6). The immunoprecipitated chromatin was eluted in 200µl elution buffer (1% SDS, 0.1M $NaHCO_3$) while rotating for 20 min at RT and decrosslinked in decrosslinking buffer (1% SDS, 0.1M NaHCO3, 1M NaCl) in a 45°C shaking heat-block overnight. In parallel, 30µl of sonicated input chromatin was decrosslinked under the same conditions. The DNA was purified over QIAquick PCR columns (Qiagen) and ten separate anti-HA or anti-GFP ChIPs were pooled over one column.

For each line, the purified DNA from ten ChIPs or 5ng of the respective input sample was used for sequencing library preparation. Libraries were end-repaired followed by the addition of 3' A-overhangs and the ligation of NEXTFlex barcoded adapters (Bio Scientific, #514122). The libraries were then amplified using the KAPA HiFi HotStart ready-mix (KAPA Biosystems) under the following conditions: 98°C for 2min; 4 cycles of 98°C for 20sec, and 62°C for 3min; 62°C for 5min. Amplified libraries were gel size-selected for ~230-330bp fragments on a 2% E-Gel SizeSelection agarose gel (Invitrogen, #G6610-02). The size-selected libraries were amplified for another 8 cycles with the KAPA HiFi HotStart ready-mix and adapter dimers were removed by purification using Agencourt AMPure XP beads (Beckman Coulter, #A63880). The library fragment size was evaluated in a Bioanalyzer High-Sensitivity run (Agilent Technologies, data not shown). Libraries were sequenced single-end for 75bp on a NextSeq500 system (Illumina) using TruSeq SR Cluster Kit v2 (Illumina) reagents.

Data were mapped with BWA samse (Version: 0.7.10-r789) to the *P. falciparum* 3D7 reference genome from PlasmoDB version 6.1 (http://www.plasmodb.org). Further data processing and analysis were performed using SAM Tools (v1.2), the BEDTools suite

(v2.20.1) and Model-based Analysis of ChIP-Seq 2 (MACS2, v 2.0.10.20130306) (Zhang et al., 2008). As PfTRF-ChIP reads were expected to map to the repetitive telomere sequences, the data was processed without removal of reads mapping to non-unique sites. Normalized bedGraph files were generated such that a value of 1 would correspond to the situation where all reads would be randomly distributed across the genome using the following formula: $n_i = r_i \times g_m/(l \times r)$ where n_i is the normalized coverage at site i, r_i is the number of fragments covering site i in the sample, g_m is the mappable genome size (22248208 bp for single-end 75 bp sequencing), l is the mean fragment size as determined in the Bionanalyzer High Sensitivity run minus adapter length and r is the number of mapped reads. These normalized files were used to generate log2 ChIP-over-input ratio tracks. Peak calling was performed using MACS2 bdgpeakcall with cut-off at 2. SignalMap browser software (v2.0, NimbleGen), the python package Fluff (v1.462) and the UCSC Genome Browser were used to visualise the data.

4.12 Supplemental References

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Chapter 5: General Discussion and Outlook

5.1 The attraction of chromosome end structures

Throughout the eukaryotic kingdom, chromosome ends are of key importance as they maintain genome integrity. They have been compared to plastic tips on shoelaces, impeding chromosomes from fraying and sticking to each other, which would eventually tangle up the organism's genetic information (Blackburn, 2005).

For many years, structural and functional analysis of chromosome ends lagged behind, which is mostly due to their repetitive nature that creates difficulties during sequence analyses. Research performed in recent years, however, accredits an exceptional biology to these regions. Interestingly, the telomeric system displays analogous features in many different organisms including *P. falciparum*. This includes (1) telomere length regulation, allowing for infinite rounds of replication; (2) epigenetic silencing, allowing for inheritable stochastic silencing and variegated gene transcription to rapidly adapt to environmental fluctuations and (3) cluster formation that is important for elevating recombination events (Louis and Becker, 2014).

The physical ends of chromosomes are composed of a tandem array of G-rich repeats that interact with multiple specialised telomeric proteins (Meyne et al., 1989; Rhodes et al., 2002). Intriguingly, this simple and common structural feature varies only slightly across different eukaryotic species, and is necessary and sufficient to preserve genome integrity by facilitating terminal DNA replication and prohibiting inappropriate fusion events. Accordingly, the length of this repeat motif strictly correlates with the cellular proliferation potential (Allsopp et al., 1992; Blackburn, 1991; Lundblad and Szostak, 1989).

Unlike the conserved and extensively studied telomeric repeat sequence, the neighbouring telomere-associated regions (TAS) display great intra- as well as interspecies variability. In virtually all eukaryotic organisms, these highly dynamic and polymorphic subtelomeric regions are composed of a mosaic of non-coding repetitive elements interspersed with and/or lying adjacent to members of multigene families. Intriguingly, these subtelomeric genes mostly code for surface-exposed proteins that mediate interactions with other organisms and/or the environment (Louis and Becker, 2014; Mefford and Trask, 2002; Verstrepen and Fink, 2009). Also, these genes reside in a transcriptionally silent state, representing another hallmark of chromosome ends. In model eukaryotes, heterochromatin at these regions is established by epigenetic mechanisms that are almost exclusively driven by the action of HP1 and TRFs. The heterochromatic state is coined by both its heritability

and the metastable and stochastic nature that allows for variable gene expression, including mutually exclusive activation of multigene family members (Louis and Becker, 2014).

In addition, chromosome ends are sequestered away from the rest of the genome by forming clusters with other chromosome ends, which are tethered to the nuclear periphery. This structural framework triggers frequent duplication and recombination events in these regions, while the rest of the genome remains unaffected. These dynamics at chromosome ends serve as breeding grounds for diversification of subtelomeric genes and allows for rapid evolutionary innovation and swift adaption to new niches or changing environments (Louis and Becker, 2014).

Even though these structural adaptations regulate vital processes and display striking parallels even between highly divergent organisms, we still lack an overall picture of the mechanisms that regulate *Plasmodium* chromosome ends. During my PhD thesis I tackled chromosome end biology in *P. falciparum* by investigating the function of PfHP1 (Chapter 2) and PfSIP2 (Chapter 3) as well as by identifying the telomere repeat-binding factor PfTRF (Chapter 4). Clearly, my work on PfHP1 has created very important new knowledge about the role of epigenetics in parasite survival and transmission. This success is in part due to the fact that HP1 proteins have been studied extensively in model eukaryotes and I could exploit this vast information for the benefit of my own research. In contrast, I was unable to generate much further insight into the function of PfSIP2 because the conditional loss-of-function approach didn't work satisfactorily, and because SIP2 has no orthologs outside the *Plasmodium* genus. My success in identifying PfTRF as the first telomere-specific factor in *Plasmodium* parasites is really exciting and paves the way for further targeted research to understand chromosome end biology and epigenetics in much more detail.

Here, I would like to discuss a few aspects of my findings that have not been discussed in depth in Chapters 2-4 and draw a working model on how these factors might interact with each other and other chromosome-end components to drive the vital processes at chromosome ends (Figure 1A-F).

5.2 PfHP1 – a master regulator of asexual progression and sexual conversion

Using a loss-of-function approach that is based on the FKBP-domain system (Armstrong and Goldberg, 2007), we show that PfHP1 is essential for mitotic proliferation and perpetuation of mutually exclusive *var* gene transcription. Both of these processes are crucial to secure the survival of *P. falciparum* parasites within the human host.

In addition and more surprisingly, we identified an intriguing role for PfHP1 in the switch from asexual proliferation to sexual differentiation that provides important insight into the unknown pathway triggering this cell fate decision (Brancucci and Bertschi et al., 2014, Chapter 2, this thesis).

5.2.1 PfHP1 and cell cycle control

In our study we also showed that depletion of PfHP1 results in a complete cell cycle arrest in 50% of the parasites (Brancucci and Bertschi et al., 2014, Chapter 2, this thesis).

In model eukaryotes, it was shown that HP1 proteins, apart from mediating heterochromatin formation, play critical roles in DNA replication and repair processes (Lomberk et al., 2006; Murzina et al., 1999; Trembecka-Lucas and Dobrucki, 2012). On the one hand, this is attributed to the chromo-shadow domain (CSD) of HP1, which mediates a variety of protein-protein interactions not only with other chromatin components but also with replication and cell cycle-related factors (Li et al., 2002; Lomberk et al., 2006). On the other hand, several independent studies showed that the HP1-mediated heterochromatic state itself is essentially involved in cell cycle control. It was shown that this repressive environment has an inhibitory effect on the activation of DNA damage response pathways, and hence relaxation of heterochromatin through the loss of HP1, consequently activates DNA damage repair mechanisms ultimately triggering cell cycle arrest (Dinant and Luijsterburg, 2009).

Analogues to model eukaryotes, the stalling of PfHP1-depeleted parasites may hence be triggered by defects in the heterochromatic state and the concomitant improper activation of DNA damage responses (Figure 1A). Interestingly, HP1-dependent heterochromatin formation at telomeres is dependent on TRFs (see paragraph 5.4.1; Figure 1D) and disruption of telomere capping also triggers DNA repair pathways ultimately halting cell cycle progression (Blasco, 2007; Garcia-Cao et al., 2004; Kanoh et al., 2005; Palm and de Lange, 2008). It is therefore tempting to assume that the cause of the observed defect in cell cycle progression of PfTRF-depleted parasites (Chapter 4, this thesis) lies within the same pathway as the block in G1/S phase transition observed in PfHP1-depleted parasites.

A highly speculative but equally interesting note is that, reminiscent to our above observation, *P. vivax* liver stage parasites are able to induce a cell cycle exit prior to schizogony. These so-called hypnozoites then can be reactivated to enter schizogony at a later time point, thereby causing a relapse of symptomatic infections (Cogswell, 1992). It will thus be very interesting to investigate whether the observed G1-arrest in our conditional *P. falciparum* mutants shares regulatory features with dormant hypnozoites in *P. vivax*. Unfortunately, such studies are currently hindered by the fact that no reliable *P. vivax in vitro* culture system is available yet.

5.2.2 PfHP1 and the silencing of the *ap2-g* locus

Our data revealed that PfHP1 depletion increased sexual conversion rate by 25-fold, with half of the parasites entering gametocytogenesis. Independently, Coleman and colleagues showed that downregulation of the histone deacetylase 2 (PfHda2) and concomitant erasure of the heterochromatic state increased sexual conversion rate by 3-fold (Coleman et al., 2014). Recent work in *P. falciparum* and *P. berghei* showed that the AP2-G transcription factor is essential for gametocyte production (Kafsack et al., 2014; Sinha et al., 2014). Our comparative transcriptional profiling demonstrated that commitment to sexual development in mid/late schizonts coincides with a specific de-repression of the *ap2-g* locus. Hence, together with the results from Coleman and colleagues, our observations show that sexual conversion in the malaria parasite is under a strong epigenetic component (Brancucci and Bertschi et al., 2014, Chapter 2, this thesis; Coleman et al., 2014).

Intriguingly, *ap2-g* is the only member of the ApiAP2 transcription factor family that is associated with and controlled by PfHP1 (Brancucci and Bertschi et al., 2014, Chapter 2, this thesis; Flueck et al., 2009). In contrast to *var* genes located in large heterochromatic domains, *ap2-g* belongs to a small group of genes that – despite being associated with PfHP1 – are located within euchromatic domains (Flueck et al., 2009). Even though *var* genes and the *ap2-g* locus are both silenced in a PfHda2- and PfHP1-dependent manner, it remains unanswered whether the silenced states of these loci are established and controlled by the same mechanisms. It has hence been speculated that the *ap2-g* locus may participate in the mutually exclusive *var* gene transcription programme (Alano, 2014; Kafsack et al., 2014).

However, it is important to note that silencing of *var* genes in *P. falciparum* is also dependent on the histone deacetylase activity of PfSir2A and PfSir2B (Duraisingh et al., 2005; Tonkin et al., 2009), as well as on the histone methyltransferase activity of PfSET2 (Jiang et al., 2013), all of which are uncoupled with gametocyte conversion. Moreover, the temporal transcription profile of *var* genes in ring stage parasites does not correlate with the transcriptional activity of the *ap2-g* locus, which is induced only after the onset of schizogony (Brancucci and Bertschi et al., 2014, Chapter 2, this thesis; Kyes et al., 2007). Taken together, this points towards two at least partially independent regulatory pathways.

Indeed, in model eukaryotes HP1-dependent repression of single euchromatic loci is established and regulated differently compared to transcriptional silencing at telomeric and pericentric regions (Greil et al., 2003; Grewal and Jia, 2007; Hwang et al., 2001; Li et al., 2002; Wallrath, 1998). In analogy to the *ap2-g* locus, some of these loci are known to code for master regulators of cellular differentiation (Fanti et al., 2003; Greil et al., 2003; Hwang et al., 2001; Jedrusik-Bode, 2013). One of the best-characterised mammalian systems for HP1-

mediated silencing of euchromatic genes represents the KRAP-KAP1 repression system (Cammas et al., 2002; Schultz et al., 2002; Sripathy et al., 2006). KRAP-KAP1 mediates transcriptional repression of a large set of euchromatic loci coding for C_2H_2-type zinc-finger transcription factors (O'Geen et al., 2007). It is proposed that KAP1 acts as a scaffold protein that coordinates the assembly of a complex consisting of histone deacetylases, histone methyltransferases and HP1. Via the interaction with the DNA-binding protein KRAB, the complex is specifically directed to euchromatic promoter regions, thereby allowing for the *de novo* establishment of a heterochromatic, repressive microenvironment at these loci (Ayyanathan et al., 2003; Ryan et al., 1999; Schultz et al., 2001, 2002). In contrast to subtelomeric regions, the transcription factor-dependent deposition of chromatin modifiers assures that epigenetic changes remain relatively short-ranged (Ayyanathan et al., 2003; Lechner et al., 2000; Schultz et al., 2001, 2002).

An alternative system was described for the regulation of the cell fate inducer IME1 in yeast. Similarly to AP2-G, targeted activation of this master regulator induces a stop in mitotic cell proliferation and propels a morphogenesis programme that eventually leads to gamete formation (Kassir et al., 1988; van Werven and Amon, 2011). As for the *ap2-g* locus, it was shown that epigenetic mechanisms play an essential role to repress *ime1* transcription (van Werven and Amon, 2011). However, in contrast to the KRAP-KAP1 system in mammals, long non-coding RNA (lncRNA) transcripts originating from the *ime1* promoter itself act as a scaffold to promote local heterochromatin formation at the *ime1* locus (Koziol and Rinn, 2010; van Werven et al., 2012).

Even though *P. falciparum* lacks KRAP-KAP1 repression systems and *ap2-g*-specific lncRNA transcripts have not been discovered so far, it will be interesting to see if conceptually similar mechanisms are responsible for the specific PfHP1-dependent silencing of the *ap2-g* locus.

5.2.3 PfHP1 and the activation of the *ap2-g* locus

We showed that a knockdown of PfHP1 leads to the activation of the *ap2-g* locus (Brancucci and Bertschi et al., 2014, Chapter 2, this thesis). However, the natural upstream pathways that eventually lead to the dissociation of PfHP1 from *ap2-g,* thereby inducing sexual conversion, remain elusive.

Committing to sexual development is key for transmission of the malaria parasite to the mosquito vector and hence essential for completion of the life cycle. However, sexual conversion antagonises asexual replication and hence uses up the parasite's own source for transmission. Therefore, balancing asexual replication and sexual conversion represents a fundamental evolutionary challenge and asks for a wise decision making pathway.

In model eukaryotes it was shown that such cell fate decisions are usually triggered via the perception of either extrinsic signals or intrinsic states to optimise resource allocation in the trade-off between growth/survival and reproduction (Lunyak and Rosenfeld, 2008; Stearns, 1992). Already in 1970, Carter and Miller demonstrated that environmental cues influence *P. falciparum* sexual differentiation (Carter and Miller, 1979). Meanwhile it is proven that environmental stress factors, such as host immunity (Buckling and Read, 2001; Talman et al., 2004), host hormones, high parasitaemia (Bruce et al., 1990; Lingnau et al., 1993) or drug treatment (Buckling et al., 1997) are associated with increased rates of sexual commitment (Dyer and Day, 2000). Moreover, it was shown that addition of reticulocyte-rich blood *in vitro* leads to an up to 10-fold increase in gametocyte production (Trager and Gill, 1992; Trager et al., 1999). It is believed that reticulocytes might secrete signals that are sensed by the parasites as beneficial condition to induce the “time-consuming” maturation of gametocyte development (Alano, 2014; Carter et al., 2013; Trager et al., 1999). Furthermore, a recent study reports that parasite populations, cultured in presence of well-defined conditioned medium (growth medium that has already used to culture cells and is nutrient depleted), increase sexual conversion rates by 40-80-fold (Brancucci et al., 2015). Two studies further suggest that cell-cell communication between parasites via extracellular vesicles also influences cell fate decisions towards sexual conversion (Mantel et al., 2013; Regev-Rudzki et al., 2013). Such extracellular vesicles hence might be implicated as factors in conditioned medium that contribute to observed increase in sexual conversion (Josling and Llinas, 2015). In summary, environmental signals seem to trigger a cascade that feeds into the pathway to ultimately modulate PfHP1-binding at the *ap2-g* locus and trigger sexual differentiation.

In mammals it was shown that upon differentiation cues, downstream signalling pathways ultimately lead to the phosphorylation of KAP1, which in turn unleashes the repressors from the scaffold to promote transcriptional activation (Kamitani et al., 2008; Singh et al., 2015). Moreover, it was shown that kinases, activated by upstream signals such as cytokines or growth factors, are directly linked with transcriptional activation via mediating targeted phosphorylation of serines at position 10 and 28 of histone 3 (H3S10ph/H3S28ph) (Baek, 2011). Intriguingly, these histone marks antagonise HP1-binding. Moreover, they recruit protein 14-3-3 that, in turn, attracts transcription factors to facilitate transcription at target loci (Baek, 2011; Prigent and Dimitrov, 2003; Schuettengruber et al., 2011; Zhang et al., 2005).

Interestingly, there is evidence for a role of kinase activation via the phorbol ester-induced, as well as the cyclic AMP-dependent signalling pathways in sexual commitment in *P. falciparum* (Doerig, 1997; Dyer and Day, 2000). Moreover, the histone modification H3S10ph and H3S28ph were recently detected in *P. falciparum* (Dastidar et al., 2013; Doerig et al., 2015). It was also shown that the histone-reader protein Pf14-3-3, containing a phosphor-

binding module, specifically binds to these histone modifications (Dastidar et al., 2013). Intriguingly, preliminary data suggest that this protein represents a key component of the PfHP1 interactome (M. Filarsky, unpublished). If these signal cascades and histone modifications are indeed involved in the eviction of PfHP1 form the *ap2-g* locus remains to be proven (Figure 1B). Nevertheless, recent data suggests that upon activation, AP2-G is able to regulate its own expression in a positive feedback loop and is hence able to ensure that cell fate decision is irreversibly (Kafsack et al., 2014; Sinha et al., 2014).

Although a challenging endeavour, it will be important to study where commitment-informing environmental cues originate from *in vivo* and which downstream signalling cascades are activated. Recently, Brancucci and colleagues succeeded in generating a readout assay for sexual conversion, which represents an excellent tool to study triggers of sexual conversion (Brancucci et al., 2015). Identification of host and parasite factors that elicit a change in conversion rate may allow for manipulating parasites to make suboptimal decisions for their fitness (Carter et al., 2013). Such knowledge might become key for maximising the success of future intervention strategies. Moreover, it is possible that such proposed environmental sensing mechanisms also influence other vital parasite decisions, such as sex ratio determination, cell cycle progression and/or *var* gene switching.

5.3 A working model for PfSIP2

Previous data suggested that PfSIP2 is essential for parasite survival (Flueck et al., 2010). I successfully generated a PfSIP2 loss-of-function mutant using the DD-system, but unfortunately this approach did not allow for novel insights into the function of PfSIP2 (Chapter 3, this thesis). Our results, however, show that PfSIP2 is only expressed during a very narrow time window of approximately 10hrs in late stage parasites, which coincides with intra-erythrocytic schizogony (Flueck et al., 2010; Chapter 3, this thesis). Moreover, in collaboration with R. Bartfai (RIMLS, Radboud University Nijmegen, The Netherlands), I showed that endogenous PfSIP2 binds to clusters of the bipartite subtelomeric promoter elements 2 (SPE2) *in vivo* (Chapter 3, this thesis). More specifically, this motif is found within the TARE2/3 region as well as in the upstream region of the promoter of *ups*B-type *var* genes (Flueck et al., 2010; Voss et al., 2003). Although I was unable to attribute any experimental evidence, the exclusive location of PfSIP2 at subtelomeric regions as well as its temporal expression profile, suggest that this protein is involved in the nucleation of subtelomeric heterochromatin, as proposed previously (Flueck et al., 2010). On the one hand, this might be achieved via the direct recruitment of chromatin remodellers by PfSIP2 to subtelomeric regions, thereby inducing a transcriptional repressive state. On the other hand, PfSIP2 might interact with the recently discovered lncRNA-TARE transcript in order to induce a repressive state at chromosome ends. The latter is discussed in the following paragraph.

5.3.1 A role for PfSIP2 in the nucleation of subtelomeric heterochromatin

Similar to yeast and higher eukaryotes, genome-wide ChIP studies highlighted that subtelomeric regions in *P. falciparum* are enriched with heterochromatin marks such as H3K9me3 and by PfHP1 (Flueck et al., 2009; Lopez-Rubio et al., 2009; Salcedo-Amaya et al., 2009). This heterochromatic state was shown to be crucial for the mutually exclusive expression of *var* genes (Brancucci and Bertschi et al., 2014, Chapter 2, this thesis). However, so far it remains largely unknown how this repressive state at telomeres/ subtelomeres is established.

The common assumption that subtelomeric and telomeric regions are transcriptionally silent was recently challenged by the finding that the C-strand of telomeres is transcribed by RNA Polymerase II, giving rise to UUAGGG repeat containing lncRNAs, termed TERRA (Azzalin et al., 2007; Feuerhahn et al., 2010; Luke and Lingner, 2009; Schoeftner and Blasco, 2008). It was shown that transcription of TERRA is initiated at subtelomeric regions through the binding of a specific transcription factor to GC-rich promoter elements (Deng et al., 2012; Nergadze et al., 2009). Therefore, apart from telomere repeat motifs, TERRA molecules also comprise subtelomeric sequence elements. It is believed that through an intimate interaction with chromatin modifiers, TERRA acts as architectural scaffold to induce the establishment of a heterochromatic state at chromosome ends (Bernstein and Allis, 2005; Koziol and Rinn, 2010; Nagano and Fraser, 2009; Tsai et al., 2010). Importantly, this may be mediated by proteins such as HP1, HMTs and HDACs, driven by the ability of TERRA to direct these factors to their site of action via the specific interaction with transcription factors and/or telomere repeat-binding factors (Azzalin and Lingner, 2015; Deng et al., 2009; Luke and Lingner, 2009; Mercer and Mattick, 2013; Tsai et al., 2010). Alternatively, guidance to target loci can as well occur by TERRA, binding to DNA as a RNA:DNA heteroduplex (Hung and Chang, 2010) .

Reminiscent to TERRA transcripts in model eukaryotes, Broadbent and colleagues reported the existence of a subtelomeric lncRNA family in *P. falciparum*. Their work revealed that these lncRNAs originate from the TARE2/3 region of 22 chromosomes and harbour an average of 15 SPE2 motifs per RNA molecule (Broadbent et al., 2011, 2015). These studies further showed that the transcription of these lncRNA-TAREs is strongly induced after initiation of DNA replication at 36hpi and shows maximal transcript abundance during erythrocyte re-invasion (Broadbent et al., 2011, 2015). Interestingly, this temporal transcription profile shows striking correlation with the expression of endogenous PfSIP2. Based on the above-mentioned findings in model organisms and the work published by Broadbent and colleagues, I propose a hypothetical and speculative working model on how PfSIP2 and lncRNA-TAREs (as well as potential interacting factors) might be involved in the

nucleation of heterochromatin formation at subtelomeres. I acknowledge that there is no direct experimental evidence for these ideas so far and many more factors might be involved in this process.

I propose that PfSIP2 and lncRNA-TAREs represent a molecular trafficking system that targets chromatin-modifying complexes to subtelomeric regions and results in the establishment of a specific epigenetic landscape at these regions. More precisely, I suggest that upon DNA replication and the concomitant loosening of the repressive state at subtelomeric regions, PfSIP2 can acquire access to SPE2 arrays in the TARE2/3 locus and initiate lncRNA-TARE transcription. In analogy to findings for TERRA function in model eukaryotes, lncRNA-TARE transcripts may act as scaffold to assemble PfHP1, H3K9me3-specific HMTs and PfHda2 into a higher-ordered complex. Presence of the SPE2 arrays within the lncRNA transcripts themselves may allow PfSIP2 to assemble into the complex. Such proposed complexes would hence be equipped on the one hand with chromatin modifying enzymes able to reprogram the epigenetic state, and on the other hand with PfSIP2, able to guide this complex to telomere-proximal regions (Figure 1C). The fact that PfSIP2 expression and lncRNA-TARE transcription temporally coincides with DNA replication and concomitant schizogony makes it plausible that these factors are involved in establishing a post S-phase epigenetic memory, allowing for proper re-establishment of a silenced state at *var* gene loci after DNA replication. This might as well be connected to the recruitment of the histone deacetylase PfSir2A in subsequent ring stage parasites (Duraisingh et al., 2005; Tonkin et al., 2009).

Further, our data indicates that PfSIP2 is only expressed after the onset of schizogony and is actively degraded by the parasite in segmented schizonts and/or merozoites (Chapter 3, this thesis). I hence propose that this specific expression pattern of PfSIP2 is sufficient to restrict the temporal transcription of lncRNAs to late stage parasites only. Alternatively, the proposed PfSIP2/lncRNA-mediated heterochromatin formation at subtelomeric regions might be responsible for shutting down transcription of lncRNA-TARE in newly formed merozoites. In such a scenario, transcription would only be reinitiated during the next DNA-replication cycle that will render the loci accessible for PfSIP2-binding and concomitant lncRNA-TARE transcription (Figure 1C).

Clearly, it will require substantial experimental efforts to test this speculative model. First, it will be important to verify a direct interaction of PfSIP2 with lncRNA-TARE by RNA gel-shift assays and/or RNA-immunoprecipitation followed by high-throughput sequencing (RIP-Seq) (Zhao et al., 2010). Preforming Chromatin RIP followed by high-throughput sequencing (ChRIP-Seq) experiments could be an interesting approach to further identify and verify genomic sites that are specifically enriched with lncRNA-TAREs (Chu et al., 2012). Another approach might be to assay putative phenotypic consequences of abrogated TARE2/3

transcription in a cell line where endogenous SPE2 binding sites at TARE2/3 are eliminated by the CRISPR/Cas9 genome-editing tool (Ghorbal et al., 2014). In order to gain insights of the function of PfSIP2, I also suggest to generate a conditional knockout mutant, using the recently established DiCre system (Collins et al., 2013) (for details see Discussion in Chapter 3, this thesis). Moreover, Co-Immunoprecipitations (Co-IPs) will also be required to uncover interaction partners of PfSIP2.

5.3.2 The role of PfTRF in the proposed model

Intriguingly, two recent studies report the finding of lncRNA transcripts in *P. falciparum* that are not restricted to TARE2/3 regions. Instead, they were found to contain telomere repeat motifs and to be associated with the entire TARE 1-6 region (Broadbent et al., 2015; Sierra-Miranda et al., 2012; Vembar et al., 2014). In model eukaryotes, it is proposed that TERRA transcripts act as scaffold to recruit and stabilise heterochromatin factors at telomeric regions via the interaction with TRFs (Deng et al., 2007, 2009; Feuerhahn et al., 2010). Hence, similar to the proposed model for PfSIP2, PfTRF might thus also be involved in guiding chromatin remodellers to telomeric and subtelomeric regions via the interaction with the recently identified lncRNA-TARE transcripts (Figure 1D, 1F). Alternatively, PfTRF might directly interact with chromatin remodellers and thereby inducing a transcriptional repressing state at telomeric and subtelomeric regions (see paragraph 5.4.1 Figure 1D, 1F).

Moreover, it is appealing to speculate that the recently identified telomere repeat-containing lncRNA-TARE transcripts might as well interact with the putative RNA-binding protein PF3D7_0605100 to promote nucleation of subtelomeric heterochromatin. This protein was affinity-purified using a telomere-repeat target sequence (Chapter 4, this thesis) and contains tandem KH-domains. Multi-KH-domain proteins are evolutionary highly conserved proteins and were shown to interact in a sequence-specific manner with single-stranded nucleic acid carrying the telomere repeat motif in model eukaryotes (Dejgaard and Leffers, 1996; Du et al., 2004, 2005; Lacroix et al., 2000; Makeyev and Liebhaber, 2002).

Moreover, it was shown that a multi-KH-domain protein contribute to the structural organisation of heterochromatin via the proper deposition of H3K9me3 and HP1 in a RNA-dependent fashion in Drosophila (Birchler et al., 2004; Du et al., 2005; Huertas et al., 2004). My preliminary data suggests that PF3D7_0605100 indeed localises to chromosome-end clusters specifically in late schizont stages (44-48hpi), which correlates with the peak expression of lncRNA-TARE and PfSIP2 (data not shown). It is hence tempting to speculate that PF3D7_0605100 is involved in placing hallmarks on replicated DNA to establish repressive heterochromatin in newly formed merozoites. A promising approach to follow up on these ideas will include verification that PfTRF and/or PF3D7_0605100 are able to

interact with the recently identified lncRNA-TARE transcripts. This could be done by using RNA gel-shift assays and/or RIP-Seq experiments.

Alternatively, PfTRF might be involved in promoting lncRNA transcription initiation at *ups*B *var* promoter elements (Figure 1F). I show that PfTRF specifically binds to interstitial telomere-repeat like elements in *ups*B *var* promoter regions (Chapter 4, this thesis). This finding is reminiscent of the role of vertebrate-specific C_2H_2-type zinc finger transcription factor CTCF that favours lncRNA transcription by binding to GC-rich elements in subtelomeric regions (Deng et al., 2012; Maicher et al., 2014; Porro et al., 2014). It remains to be seen whether lncRNA transcription is initiated at PfTRF binding sites. However, if indeed such lncRNA transcripts exist, they might represent subtelomeric equivalents to the GC-rich lncRNAs found at internal *var* genes, which are involved in both *var* gene activation and the silencing of neighbouring genes (Figure 1F) (Panneerselvam et al., 2011; Wei et al., 2015). The PfTRF-loss-of function mutant (Chapter 4, this thesis) might serve as a good tool to study potential changes in lncRNA-TARE transcription upon PfTRF depletion.

5.4 PfTRF - Identification of the first telosome component in *P. falciparum*

TRFs, as initiators and mediators of the telosome complex, play essential roles in preserving chromosome integrity and regulating chromosome length (Xin et al., 2008). My work reports about the successful identification of a C_2H_2-type zinc-finger protein as the *P. falciparum* telomere repeat-binding factor (PfTRF). Intriguingly, this is the first example of a C_2H_2-type TRF in eukaryotes, suggesting that *Plasmodium* parasites employ an evolutionary divergent molecular complex to preserve telomere function. This obvious difference notwithstanding, our analysis of a loss-of-function mutant uncovered that the PfTRF shares its role in telomere length regulation with model TRFs (Chapter 4, this thesis). However, if PfTRF may also share similar roles as model TRFs in heterochromatin formation and/or spatial chromosome organisation is discussed in the following paragraphs.

5.4.1 A role for PfTRF in heterochromatin formation

In many different organisms it was observed that telomeres, apart from protecting chromosome integrity, are also involved in the enrichment of heterochromatic marks at chromosome ends that, in turn, impacts transcription of nearby subtelomeric genes (Baur et al., 2001; Gottschling et al., 1990). This so-called telomere position effect (TPE) was shown to be dependent on TRFs. While in *S. cerevisiae*, RAP1 mediates the recruitment of silencing information regulator (SIR) components to telomeres, which consequently leads to the spreading of heterochromatin into subtelomeric regions (Kueng et al., 2013; Moretti et al.,

1994; Ottaviani et al., 2008; Rusché et al., 2002), it was shown that TRFs of other model eukaryotes play essential roles in establishing a HP1-dependent heterochromatic state at subtelomeric region via the recruitment of HTMs that tri-methylate H3K9 and thereby induce HP1-binding (Benetti et al., 2008; Kanoh et al., 2005; Koering et al., 2002; Nakayama et al., 2001; Schoeftner and Blasco, 2010; Zhang et al., 2008). As discussed in paragraph 5.3.1 and 5.3.2, TERRA molecules acting as scaffold, play at least partially an important role in this process (Luke and Lingner, 2009).

In *P. falciparum*, the close proximity of *var* members to chromosome ends suggests a dominant impact of the TPE on the regulation of these important virulence genes. *var* gene regulation may actually share characteristics with the mutually exclusive expression of variant surface glycoproteins (VSG) in trypanosomes. Here, the knockdown of an intrinsic component of the telosome complex, TbRAP1, led to simultaneous de-repression of subtelomerically located *vsg* genes (Yang et al., 2009). First evidence that TPE does exist in *P. falciparum* came from a study demonstrating that transcription of a gene located adjacent to telomere repeats is lowered by about 50-folds as compared to the identical gene localised distant from telomeres (Figueiredo et al., 2002). The mechanisms that underlie TPE establishment, however, remain largely unknown. Apart from HP1/H3K9me3, an analogous function has been proposed for *P. falciparum* origin recognition complex subunit 1 (PfOrc1) and PfSir2A, which both bear striking resemblance to *S. cerevisiae* SIR components, (Mancio-Silva et al., 2008). The fact that both PfOrc1 and PfSir2A localise to subtelomeric regions (Deshmukh et al., 2012; Freitas-Junior et al., 2005; Mancio-Silva et al., 2008) and that PfSir2A has further been implicated in subtelomeric *var* gene silencing (Duraisingh et al., 2005; Merrick et al., 2010; Tonkin et al., 2009), provides additional evidence for this scenario. Moreover, PfSir2a is attributed with a role in telomere length control and genome stability, suggesting that this protein indeed is a member of the same interaction network driving telomere function (Merrick et al., 2015; Tonkin et al., 2009).

Importantly, with the identification of PfTRF as the first telosome component in *P. falciparum* it will now be possible to test if PfTRF is directly involved in telomeric/subtelomeric heterochromatin formation and to resolve the mechanisms underlying TPE and its effects on *var* gene silencing (Figure 1D). I hence suggest conducting Co-IP experiments to identify further telosome components and to resolve if PfTRF – analogous to *S. cerevisiae* – indeed interacts with PfSir2A and PfOrc1 to establish a repressing environment at telomeric regions. Further, I propose analysing potential changes in genome-wide PfHP1 distribution by ChIP-Seq experiments in the PfTRF-loss-function mutant, to unravel a potential role of PfTRF in mediating PfHP1-dependent gene silencing. Moreover, this should be complemented by analysis of the transcriptional profile of subtelomeric genes, especially of *var* genes, in the PfTRF loss-of function mutant.

5.4.2 A role for PfTRF in spatial chromosome organisation

Over the past decade, extensive research in model eukaryotes revealed that chromosomes are non-randomly distributed, but rather occupy preferential positions within the nucleus. It was shown that chromosome ends form cluster with other chromosome ends, which are tethered to the nuclear periphery (Feuerbach et al., 2002; Hozé et al., 2013; Wong et al., 2013; Zimmer and Fabre, 2011).

In *P. falciparum,* chromosome ends as well are organised into 4-7 perinuclear foci (Figueiredo et al., 2002; Freitas-Junior et al., 2005). Since all but the single active *var* gene are found within these foci, spatial positioning is believed to play a major role in the establishment of a transcriptionally repressive state, and to represent a key component of mutually exclusive *var* gene expression (Issar et al., 2009; Lopez-Rubio et al., 2009; Ralph et al., 2005; Volz et al., 2010) Independent of gene regulatory mechanisms, it is believed that the spatial positioning of telomeric clusters at the nuclear periphery as well provides a structural framework for gene conversion events (Freitas-Junior et al., 2000).

It is however largely unknown how *Plasmodium* telomeres become tethered and physically anchored to the nuclear periphery. Evidence exist that telomere associated regions (TAS) are important for the formation and stability of telomere clusters in *P. falciparum* (Figueiredo et al., 2002). Moreover, Zhang and colleagues recently showed that a highly conserved central repeat region of the *var* gene intron is a major effector in the perinuclear tethering process and that Pfactin1 is a prominent member of the protein complex binding to this region (Zhang et al., 2011).

Extensive research in model eukaryotes unveiled that TRFs provide a versatile tool in guiding movement and attachment of telomere clusters to the nuclear periphery. On the one hand, the prominent role of TRFs in the 3D distribution of telomeres is attributed to their capability to dimerise/multimerise and/or stabilise DNA G-quadruplex structures between telomeres, thereby provoking telomere cluster formation. On the other hand, it was shown that TRFs directly interact with lamins – a major structural component of the nuclear envelope – and hence are able to directly link telomeres to the nuclear periphery (Wood et al., 2014). Lamins, on their part, were shown to interact with the CSD of human HP1alpha and HP1beta, thereby provoking a repressive heterochromatic environment at the nuclear periphery (Kourmouli et al., 2000; Makatsori et al., 2004; Polioudaki et al., 2001; Pyrpasopoulou et al., 1996; Ye and Worman, 1996; Ye et al., 1997). Accordingly, mutations in lamin-coding genes are linked to defects in the spatial positioning of telomeres and can result in decreased genomic stability (Gonzalez-Suarez et al., 2009).

Even though lamins are metazoan-specific, it is still tempting to speculate that – in analogy to findings in model eukaryotes – PfTRF might play a crucial role in the spatial positioning of

chromosomes within the perinuclear space, thereby contributing to the mutually exclusive expression of *var* genes (Figure 1E). Evidence exists that similar mechanisms might indeed operate in *Plasmodium* as the so-called Perinuclear Repressive Centres (PERC), which contain silenced subtelomeric domains, are enriched in PfHP1, PSir2A, PfOrc1 as well as Histone Methyltransferase PfSET3 (Flueck et al., 2009; Freitas-Junior et al., 2005; Lopez-Rubio et al., 2009; Mancio-Silva et al., 2008; Pérez-Toledo et al., 2009). Studying telomere cluster formation in the PfTRF loss-of-function mutant by DNA-FISH using a telomere probe might be used to unravel a potential role for PfTRF in spatial positioning of telomeres in *Plasmodium* parasites. If so, Co-IP experiments, using PfTRF as bait, may be used to identify interaction partners at the nuclear periphery that contribute in regulating this important process. However, visualisation of chromosome-end clusters in the PfTRF-loss-of-function mutant by anti-PfHP1 antibodies in preliminary IFA experiments did not suggest aberrant formation of perinuclar foci in absence of PfTRF (preliminary data, not shown). Nevertheless, further experimental proof is required.

Additionally, it was shown that TRFs not only play an important role in the spatial positioning of chromosome-end clusters during the interphase, but as well have a major function in the proper segregation of genetic material during mitosis and meiosis by allowing for proper alignment of sister chromatids and homologous chromosomes, respectively (Amelina et al., 2015; Canudas et al., 2007; Cooper et al., 1998; Lan et al., 2014; Liebe et al., 2004; Prime and Markie, 2005). Since we observed a defect in proper cell cycle progression in PfTRF-depleted parasites, this might indicate that PfTRF shares similar mechanisms (Figure 1E). Having said this, tackling a potential role of PfTRF in the meiotic stages in the mosquito midgut would be a further very interesting approach to pursue.

5.4.3 A role for PfTRF at subtelomeric binding sites

The unexpected occupancy of PfTRF at interstitial telomere repeat sequences (ITS) in *var* gene promoters (Chapter 4, this thesis) suggests a role for this protein in virulence gene regulation. As proposed, PfTRF might be involved in the direct recruitment of histone remodellers to telomeric and subtelomeric regions (paragraph 5.4.1, Figure 1F (i)), and/or might promote recruitment of the silencing machinery to telomeric and subtelomeric regions via the interaction with scaffold lncRNA (paragraph 5.3.2; Figure 1F (ii)), thereby inducing transcriptional repression of *var* genes. Alternatively, PfTRF might be involved in lncRNA transcription at ITS thereby inducing transcriptional repression of *var* genes (paragraph 5.3.2; Figure 1F (iii)). Moreover, as discussed in Chapter 4, telomeres capped with PfTRF might loop back and interact with ITS, thereby inducing a telomere-position effect over long distance (TPE-OLD) (Figure 1F (iv)) (Robin et al., 2014; Zaman et al., 2015).

Intriguingly, however, research in model organisms revealed that such interstitial telomere like sequences (ITSs) might as well act as hotspots for chromosome breakage, recombination and rearrangements during meiosis (Blitzblau et al., 2007; Kilburn et al., 2001; Lin and Yan, 2008). It is believed that the high GC content of ITSs promotes G-quadruplex formation, thereby facilitating recombination and genomic exchange (Maizels, 2006).

Reflected by the high recombination rate, *P. falciparum* subtelomeric regions represent a hotspot for genomic exchange (Scherf and Mattei, 1992). These mitotic as well as meiotic recombination events are crucial for generating antigen sequence diversification (Freitas-Junior et al., 2000; Scherf and Mattei, 1992). The elevated recombination rate can at least be partially attributed to the spatial positioning of telomeric clusters at the nuclear periphery, as well as to TAS-mediated paring of chromosome ends, which provide a structural framework for gene conversion events (Figueiredo et al., 2002). Nevertheless, it is tempting to speculate that, similar to findings in model eukaryotes, ITSs may enhance recombinational activity within *var* gene arrays to generate the virtually unlimited diversity of antigenic and adhesive phenotypes observed for PfEMP1 (Figure 1F).

Taken together, removal of one or several ITS sites in *ups*B promoters using the genome-editing tool CRISPR/Cas9 (Ghorbal et al., 2014) will aid to understand the contribution of PfTRF binding to these internal telomere like sequences in *var* gene silencing and/or gene conversion events.

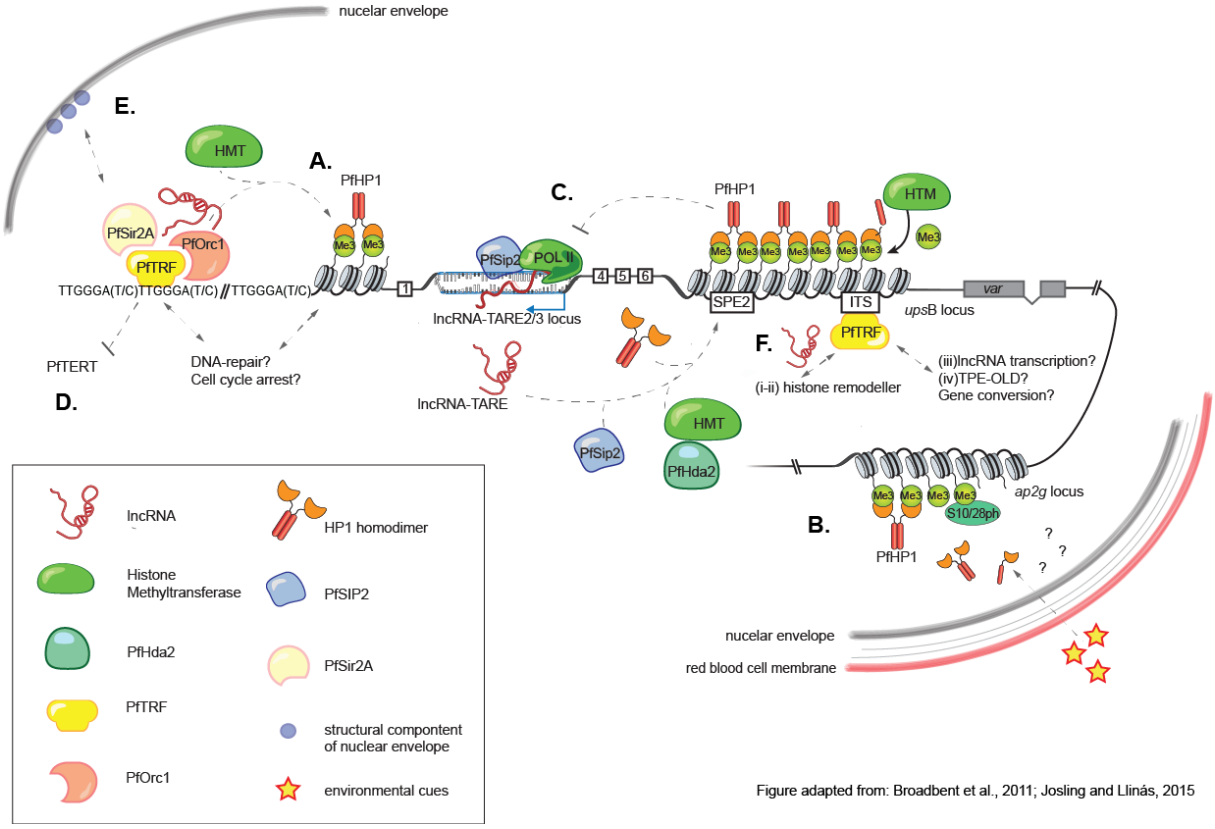

Figure 1. Schematic display of a speculative model on how PfHP1, PfSIP2 and PfTRF interact at chromosome ends in *P. falciparum*.

(A) PfHP1-mediated heterochromatic state at telomeres and subtelomeres is crucial for mutually exclusive *var* transcription (Chapter 2) and might control G1/S-phase transition via the regulation of DNA repair mechanisms (paragraph 5.2.1). (B) PfHP1 mediates repression of the *ap2-g* locus (paragraph 5.2.2), and environmental cues might induce de-repression of the *ap2-g* locus via signalling pathways leading to H3S10/28ph, thereby inducing sexual conversion (paragraph 5.2.3). (C) A speculative model how PfSIP2 and lncRNA-TARE might mediate nucleation of subtelomeric heterochromatin at TARE2/3 and *ups*B promoter elements (paragraph 5.3.1) (D) PfTRF might be involved in the establishment of a heterochromatic state at chromosome ends by the recruitment of PfSir2A, PfOrc1 and/or HMT and PfHP1 (paragraph 5.4.1). The recruitment of histone remodellers might also be directed by lncRNA acting as scaffold (paragraph 5.3.2). The established heterochromatic state might regulate virulence gene expression and preserves chromosome integrity (paragraph 5.4.1 and paragraph 5.2.1). PfTRF regulates telomere length probably via regulation of the telomerase enzyme PfTERT and/or T-loop formation (Chapter 4, this thesis). (E) PfTRF might be involved in perinuclear cluster formation and/or proper segregation of chromosomes during mitosis/meiosis (paragraph 5.4.2). (F) PfTRF binding at ITS might promote transcriptional repression of *var* genes by (i) the direct recruitment of the silencing machinery; and/or (ii) recruiting histone remodellers by interaction with scaffold lncRNA (paragraph 5.3.2); and/or (iii) initiating lncRNA transcription (paragraph 5.3.2); and/or (iv) promoting loop formation over long distance (TPE-OLD) (Chapter 4, this thesis). Moreover, PfTRF binding at ITS might anticipate gene conversion events (paragraph 5.4.3).

5.5 Future directions

The *de novo* identification of PfTRF (PF3D7_1209300) as an important component of *P. falciparum* telomeres provides important new insight into the mechanisms underlying genome maintenance in malaria parasites. This newly acquired knowledge provides an excellent starting point to resolve missing components of the interaction network at telomeres that is not only essential for parasite propagation but most probably also plays a crucial role in antigenic variation. Awareness of the fact that *Plasmodium* parasites employ an evolutionary divergent molecular mechanism to preserve telomere function is highly relevant and might open up new routes for future efforts in developing anti-disease strategies for this deadly human pathogen.

The identified role for PfHP1 in triggering sexual commitment represents key information on how malaria parasites regulate this differentiation process. This knowledge will serve as an excellent starting point to resolve upstream pathways and to ultimately tackle the longstanding question about how this cell cycle switch is triggered. In addition, the induction of a gametocyte hyper-conversion event in PfHP1 loss-of-function mutants may be used to produce synchronous populations of sexual stages that may feed drug-screening platforms aiming to develop transmission intervention strategies.

I am hopeful that the work and thoughts presented in my thesis will greatly support studies that aim at resolving the mechanistic details of (i) telomere biology, (ii) epigenetic virulence gene regulation and (iii) the regulatory pathway that leads to sexual conversion in *P. falciparum* and other malaria parasites. Clearly, such knowledge will be highly relevant for our understanding of malaria pathogenesis, immune evasion and transmission and I am confident it will help moving research towards the long-desired goal of subverting malaria.

5.6 References

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