Novel Insights into the Epigenetic Regulation of *Plasmodium falciparum* Virulence Genes

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Nicolas Michel Beat Brancucci

von Courfaivre (JU)

Basel, 2013

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

_____

Prof. Till S. Voss und Prof. Kirk W. Deitsch

Basel, den 12. November 2013

_____

Der Dekan
Prof. Dr. Jörg Schibler

Table of Contents

Summary

Plasmodium falciparum malaria still places a major health burden upon the developing world. The high virulence of this parasite is linked to the variegated expression of single *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) variants that are encoded by the *var* gene family. Most *var*s and other virulence genes implicated in host-parasite interactions are found within transcriptionally inert chromatin domains at the nuclear periphery and are associated with heterochromatin protein 1 (PfHP1). Mutually exclusive gene activation relies on the escape of a single *var* locus from this repressive environment into a transcriptionally competent perinuclear zone and non-coding elements play an essential role in this process. The exact mechanisms involved in singular gene choice, however, remain elusive.

Using a transfection-based approach we identified *cis*-acting regulatory promoter elements, namely an upstream activating sequence and a mutual exclusion element (MEE), which are essential for *var* gene induction and singular locus recognition, respectively. Interestingly, in absence of the MEE, active *var* promoters are no longer introduced into the mutual exclusion programme. A yet unknown nuclear factor binds specifically to the MEE and I consider this interaction to be a prime candidate for the regulation of singular *var* activity. In addition, the MEE regulates PfEMP1 expression post-transcriptionally. On the mRNA level, this element is able to drastically reduce translation in an autonomous manner.

In an independent project, I generated a conditional PfHP1 loss-of-function mutant to functionally characterise this epigenetic regulator. After inducing PfHP1 depletion during early intra-erythrocytic development, parasites progress normally through schizogony but do not enter mitosis in the subsequent generation. Detailed analysis of these PfHP1-deprived parasites revealed four striking phenotypes: (1) they show a massive de-repression of PfHP1-controlled virulence genes, including the entire *var* repertoire; (2) over 50% of the cells in the parasite population represent viable early stage gametocytes that complete sexual development in absence of PfHP1; (3) this high conversion rate is linked to the induction of an *apiap2* TF family member during schizogony of the previous cell cycle; (4) the non-gametocyte cells represent asexual parasites that are reversibly arrested prior to enter S-phase and mitosis. We could thus identify essential roles for PfHP1 in gene silencing, cell cycle progression and showed for the first time that the mechanism of sexual conversion involves a strong epigenetic component.

1. Introduction

1.1. Malaria

Malaria, caused by apicomplexan parasites of the genus *Plasmodium*, continues to be one of the world`s most pressing human health problems. The most virulent out of five species infecting humans, *P. falciparum*, is responsible for the majority of over 600`000 lethal malaria cases annually[1]. The highly complex life cycle of this parasite encompasses several obligate developmental stages in humans and the Anopheles mosquito vector, and involves both intracellular replicative forms (intra-hepatocytic and intra-erythrocytic schizonts) and extracellular invasive forms (merozoites, sporozoites, ookinetes). The injection of *P. falciparum* sporozoites during the mosquito blood meal is followed by cycles of asexual replication within human liver cells (exo-erythrocytic schizogony) resulting in the release of thousands of merozoites into the blood stream. The subsequent schizogony within red blood cells (RBCs) is responsible for all symptoms associated with malaria and is characterized by repeated rounds of erythrocyte invasion, parasite multiplication and the consequent rupture of infected host cells. During this intra-erythrocytic developmental cycle (IDC), a small subpopulation of cells switches from asexual replication to the formation of gametocytes[2]. These sexual precursor cells represent the only parasite stage able to infect the Anopheles insect vector. Hence, parasite transmission from the human host to the mosquito essentially depends on persistent blood stage infection as a source for the generation of transmissible forms. To establish chronic infection, parasites export erythrocyte membrane protein 1 (PfEMP1) to the surface of parasitized RBCs. This integral membrane component mediates binding to a variety of host cell receptors on endothelial cells and uninfected erythrocytes[3–5]. As a consequence, parasites sequester away from peripheral blood circulation thus preventing phagocytic clearance in the spleen. The resulting formation of erythrocyte aggregates in the microvasculature of various organs is directly linked to severe outcomes, including cerebral and placental malaria[6]. Although the process of cytoadherence supports chronic infection, surface exposure of PfEMP1 is also problematic from the parasite point of view as it renders infected RBCs (iRBCs) susceptible to antibody-mediated defence mechanisms. To avoid this, *P. falciparum* employs antigenic variation of PfEMP1, which is mediated through a complex interplay of numerous epigenetic components. Despite our emerging knowledge in this field we are only now beginning to understand the underlying regulatory mechanisms that form the basis for this most efficient survival strategy of *P. falciparum*.

1.2. General Aspects of *Plasmodium* Gene Regulation

The complex life cycle of *Plasmodium* parasites requires a precise and highly coordinated control of gene expression. As in all eukaryotic organisms, transcription of protein-coding genes is induced by RNA Polymerase II (RNAPolII) at the core promoter region[7–9] and can be enhanced or repressed by specific *cis*-acting DNA/protein interactions[10]. To date, *Plasmodium* RNAPolII has not been extensively analysed. Interestingly, however, the essential C-terminal domain of this enzyme was found to be variable not only between species but, as in the case of *P. falciparum*, also within different isolates of the same parasite[11]. Recruitment of RNAPolII is aided by a set of general transcription factors, most of which have been identified in the malaria parasite[12,13]. This first regulatory checkpoint was long considered to dominate *P. falciparum* gene expression. Indeed, more than half of all genes display a temporal activity profile over the 48 hour IDC[14–17], suggesting that transcripts are only produced when their encoded gene products are required. However, a number of subsequent studies found transcription to be in loose correlation with corresponding protein levels only[17–26], implying that post-transcriptional activities significantly contribute to the control of protein synthesis. Further, sophisticated epigenetic mechanisms are operational in *P. falciparum*. These are known to be involved in mediating clonally variant gene expression (CVGE)[27–31], the driving force behind phenotypic diversity and antigenic variation.

The work presented here focuses on the epigenetic strategies used by malaria parasites to establish and maintain CVGE. Not surprisingly, we observed that these are inter-connected with other layers of gene regulation including the activity of specific transcription factors (TFs) and post-transcriptional control mechanisms.

1.2.1. Specific Transcription Factors

The proteome of *P. falciparum* reveals a striking paucity of well-known DNA-binding domains that flag transcriptional regulators found in other eukaryotic organisms[12,32]. In 2005, Balaji *et al*. discovered a novel family of putative TFs that are conserved in and specific to apicomplexan parasites (ApiAP2)[33]. They are characterised by one or more copies of the AP2-integrase DNA-binding-like domain. A protein-binding microarray assay demonstrated that a large number of these AP2 domains bind specifically to promoter elements found upstream of selected *P. falciparum* genes[34,35]. Whereas most ApiAP2 factors await functional characterisation, the three family members AP2-O, AP2-Sp and AP2-L (orthologs of PF3D7_1143100, PF3D7_1466400 and PF3D7_0730300, respectively), were shown to exert important functions in mosquito and liver stages of the rodent malaria parasite *P. berghei*.

While AP2-O activates transcription of numerous ookinete-specific genes that are essential for the development into oocysts[36], genomic deletion of *ap2-sp* and *ap2-l* blocks subsequent sporozoite formation within this cyst[37] and liver stage development[38], respectively. AP2-O and AP2-Sp appear to be recruited through particular *cis*-acting elements enriched in the upstream regions of ookinete- and sporozoite-specific target genes[36,37]. These findings unearthed an unprecedented role for ApiAP2 proteins in the control of parasite differentiation and development. Expression of all three factors in *P. falciparum* blood forms indicates that they may also exert IDC-related functions[14,16,39]. In fact, most *apiap2* members are transcribed during intra-erythrocytic *P. falciparum* development. They are induced at distinct time points along the asexual replication cycle[14–17]. This finding led to the hypothesis that ApiAP2 factors may represent master transcriptional regulators of the IDC[40]. With only 27 family members, however, their individual regulatory power might be too limited to explain the complexity of the *P. falciparum* blood stage transcriptome. Consistent with the occurrence of multiple ApiAP2 binding sites in the upstream region of many genes[35], it has been argued that parasites may overcome this restriction by using combinatorial gene regulation[35,41].

In addition to the specific role in gene activation, there is evidence that ApiAP2 proteins are also assigned to more general functions. Specifically, another family member, PfSIP2 (PF3D7_0604100), was shown to bind hundreds of distinct genomic target sites, most of which are located within subtelomeric and transcriptionally silent domains on all chromosomes[42]. Many of these SPE2 motifs are found upstream of the PfEMP1-encoding *var* genes. Since overexpression experiments revealed only minor effects on global transcription, Flueck *et al.* suggested that PfSIP2 does not act as a typical TF but rather fulfils general functions in regulating heterochromatin formation and genome integrity[42]. Interestingly, the AP2 domains of PfSIP2 orthologs are well conserved also among *Plasmodium* species that do not express PfEMP1, which provides further circumstantial evidence for a non *var*-specific role of this DNA-binding factor.

To date, there are only two additional specific TFs described outside the ApiAP2 family in *P. falciparum*. PfMYB1 (PF3D7_1315800), characterised by a Myb-like DNA binding domain, was shown to interact with a number of cell cycle-specific genes and has been suggested to play an essential role in intra-erythrocytic parasite development[43,44]. Further, recent efforts revealed that overexpression of PREBP (PF3D7_1011800), a protein containing four K-homology domains, is able to enhance transcriptional activity of an episomal target promoter[45].

1.2.2. Post-Transcriptional Regulation

5` untranslated regions (5` UTRs) of parasite transcripts are extraordinarily long compared to those in other eukaryotes[10]. Hence, the regulatory potential of these mRNA leader sequences is substantial. Indeed, several functional mapping studies revealed prominent effects on reporter gene activity upon manipulation of selected upstream regions. Often, however, it remains obscure if the observed *cis*-acting activities arise from alterations at the transcriptional or post-transcriptional level[10]. This can mainly be attributed to the fact that measuring crude transcript abundance does not suffice to draw final conclusions about whether mRNA features or transcription initiation rates are responsible for altered reporter activity.

Once transcripts are synthesised, mRNA processing and decay rates determine the steady state levels that can be translated into functional protein. A report by Shock and colleagues demonstrated that the stability of *P. falciparum* transcripts generally increases during the time course of the IDC[46]. Moreover, while functionally related genes showed similar decay rates, members of separate ontology groups differed in the dynamics at which their transcripts are degraded. These findings indicate a specific contribution of transcript half-life in gene regulation; the underlying mechanisms, however, have yet to be defined. Interestingly, genes coding for CCCH-type zinc finger proteins – common regulators of mRNA stability and translation[47] – are over-represented in the *P. falciparum* genome and thus represent compelling candidates for the control of RNA-decay rates[12].

Additionally, malaria parasites make use of translational repression; a strategy that allows for the fast adaptation to environmental changes. Most prominently, pre-synthesised transcripts in the transmissible gametocyte and sporozoite stages were shown to facilitate rapid stage conversion upon the change of host. Gametocytes in the human blood circulation, for instance, transcribe mRNA species essential for subsequent ookinete formation. These transcripts are silenced and stored within ribonucleoprotein complexes and are only translated after parasites reach the insect vector. Translational repression in gametocytes is target-specific and involves a conserved U-rich element found in either the 5` or 3` UTR of ookinete-specific genes[23,48]. By contrast, in salivary gland sporozoites, phosphorylation of eukaryotic initiation factor eIF2α prevents parasites from pre-mature development into liver-stages by repressing translation on the global scale[49].

Further means for gene regulation in *P. falciparum* include the use of upstream open reading frames (uORFs) and the production of non-coding RNA species[50–54]. These control

layers were shown to have a direct impact on the regulation of *var* genes and PfEMP1 expression and will thus be discussed on a later occasion.

1.3. PfEMP1 and the *var* gene family

Soon after invasion, parasite derived antigens are presented at the erythrocyte surface and hence represent possible immune response targets. Amongst these, the highly polymorphic PfEMP1, encoded by individual *var* gene family members, appears on the RBC surface approximately 18 hours post invasion onwards[55]. Four distinct PfEMP1 functions have been attributed to the protein`s important role in *P. falciparum* pathogenesis. These are antigenic variation[56], cytoadherence[57], rosetting (binding to uninfected erythrocytes)[3] and regulatory activities on host immune cells[58–60].

Typically, PfEMP1 proteins are composed of two to seven DBL (Duffy binding like) and one or two CIDR (cysteine-rich inter-domain region) receptor-like domains[61]. PfEMP1 variants not only differ in their antigenic properties, they can also be separated with regards to intrinsic adherence traits. Hence, besides allowing for immune evasion, phenotypic variation of PfEMP1 results in altered binding properties of parasitized RBCs, which in turn represents an important determinant of disease severity[62]. CIDR domains are often involved in binding to CD36 on endothelial cell lineages, which is considered as the primary interaction responsible for sequestration[63]. The less commonly observed binding to ICAM-1 is associated with the emergence of cerebral malaria, a frequent fatal consequence of an infection with *P. falciparum*[64,65]. Moreover, pregnancy-associated malaria is caused by the interaction of a particular PfEMP1 variant (VAR2CSA) with placental chondroitin sulphate A (CSA)[66,67].

1.3.1. *var* Genes and their Chromosomal Context

61 *var* genes are found in the 22.8 Mb haploid genome of *P. falciparum* reference strain 3D7[68]. They follow a general genomic distribution pattern, occupying the highly polymorphic chromosome end regions and a few chromosome central areas[68–71]. The overall subtelomeric organisation of the 14 chromosomes is conserved and characterised by large regions of homologous DNA that, in addition to telomeric repeats, consists of a mosaic of six telomere-associated repeat elements (TAREs)[68]. Each chromosome end typically contains one to three *var* genes located within a context of numerous members of other multigene families including *stevor*, *rif* and *Pfmc-2tm*[68]. Frequent recombination events in subtelomeric regions contributed substantially to a virtually limitless diversity in *var* sequences[72], which reflects the selective pressure acting on this immunodominant factor.

var genes are characterised by a two exon structure[69,73]. Exon I codes for the highly polymorphic extracellular portion and the transmembrane domain of PfEMP1 and is diverse with respect to both length and sequence[74]. In contrast, exon II is conserved and encodes the intracellular acidic terminal segment (ATS) that anchors the protein to parasite-induced structures, the so-called knobs, underneath the iRBC membrane[75–77].

Five different 5` upstream (*ups*) regions are associated with *var* genes; they are grouped according to sequence similarities into *upsA*, *B*, *C*, *D* and a unique *upsE* sequence[78]. Whereas the *upsC* region is exclusively associated with chromosome central *var* clusters, *A*- and *B*-type promoters control subtelomeric *var* members that are often arranged tail-to-tail with the *upsA* gene being transcribed towards the telomere. Furthermore, some *B*-type *var* genes locate adjacent to chromosome internal *var* clusters and are sub-grouped into two *ups* types (*B/A* and *B/C*) that share characteristics of either *A*- and *B*- or *B*- and *C*-type genes[79]. In contrast to these hybrids, the unique *upsE* (PF3D7_1200600) and *upsD* (PF3D7_0533100) sequences formed independently[79]. While the *upsE* promoter controls *var2csa* transcription (in CSA binding parasites), the *D*-type upstream region drives expression of a constitutively active pseudogene (PF3D7_0533100)[80] and thus falls outside the strict control of *var* gene transcription described below.

1.4. Epigenetic Regulation of *var* Gene Transcription

Antigenic variation of PfEMP1 strictly depends on mutually exclusive *var* gene expression, whereby only a single family member is transcribed by individual parasites at any time[81]. Regulation occurs in a developmentally controlled manner and is achieved *in situ* at the level of transcription initiation by RNAPolII[81,82]. It was shown that singular *var* gene activity solely depends on non-coding elements at each *var* locus – the 5` upstream sequences and the *var* gene intron[83–87] – and is independent of antigen production[88]. A number of recent studies identified a central contribution of various epigenetic mechanisms to regulate this process (described in detail below)[89]. One other important aspect is that *P. falciparum* chromosome ends are physically clustered at the nuclear periphery[72,90]. As a consequence, subtelomeric *var* genes inherently locate to the perinuclear space that is linked to enhanced transcriptional silencing in other eukaryotic organisms[91]. Interestingly, this spatial association was also demonstrated for chromosome internal *var* clusters[84,92]. The process of *var* gene activation involves nuclear re-positioning of a formerly silenced locus into an active zone, indicating the existence of a specialised transcriptionally competent perinuclear compartment devoted to singular *var* expression[84,92–96].

Interestingly, the proteins encoded by members of other virulence gene families are also expressed in a clonally variant manner[97–99] and exported to the infected erythrocyte[100,101]. The strict mutually exclusive nature of PfEMP1 expression, however, renders the *var* family a special case[102]. Here, CVGE is studied best and was shown to involve the action of various regulatory components, most of which are directly linked to epigenetic functions.

1.4.1. *var* Upstream Regions and the Role of the Intron

Transfection experiments revealed that the activity of an episomal *upsC* sequence is sufficient to silence the entire repertoire of endogenous *var* genes[84]. Prior to induction, episomal promoters are transcriptionally repressed and have no influence on other members of the gene family[84–86,103]. Hence, each *var* promoter appears to be equipped with regulatory elements allowing for its participation in the processes of silencing, activation and the associated phenomenon of mutually exclusive recognition. Indeed, unknown *trans*-acting factors were found to interact specifically with highly conserved sequence motifs; the SPE1 and CPE elements in subtelomeric and chromosome central *ups* regions, respectively. Expression of these proteins coincides with transcriptional inactivation of their target loci in trophozoites, suggesting an involvement in *var* gene repression[104]. However, the exact functional role of these interactions awaits experimental confirmation.

Another non-coding element, the *var* intron, was found to cooperate in *cis* with the *ups* region to mediate gene silencing[83,86,87]. The intron itself represents a bi-directional promoter[83] and corresponding transcriptional activity is essential for inclusion of the *ups* region into the programme of singular *var* gene choice[88,96]. Interestingly, intron-derived non-coding transcripts localise to a distinct perinuclear area where they physically associate with chromatin[50]. Generally, there is increasing evidence for the involvement of non-coding RNA species in *P. falciparum* gene regulation but the underlying mechanisms are largely unexplored[52,53].

Recent work by the Deitsch laboratory reinforces the finding that a strict one-to-one pairing between the upstream region and the intron is required for *var* gene silencing[103,105]. However, the same repressive effect can be achieved by coupling an *ups* sequence with activities from other, *var*-unrelated promoters[96]. This finding indicates that transcription itself, rather than a specific DNA element within the intron, is necessary for silencing. Regardless of the interaction partner, transcriptional inactivation of a *var* promoter was found to depend on transition through S-phase[83]. It is hence likely that the function of the intron has an epigenetic

component. Yet, it remains to be defined how information of the intron feeds back into silencing of the *ups* region.

Whatever this bridging mechanism may be, *var* upstream sequences are well known to represent central regulatory elements in the epigenetic control of *var* gene expression. Differential post-translational histone modifications in these regions are linked to either the active or silenced state of *var* genes[95,106,107]. They are stably inherited over several generations and were shown to be involved in bookmarking the poised state of an active *var* gene during non-transcribed parasite stages[106].

1.4.2. Histones at *var* Loci

Generally, different sets of genes can be associated with either histone 3 that is tri-methylated (H3K9me3) or acetylated at lysine 9 (H3K9ac). The H3K9me3 mark is evolutionarily conserved and characteristic for chromosomal domains with restricted access to the transcription machinery. At target loci, DNA is often packed into the highly condensed structure of heterochromatin and H3K9me3 provides a platform for the recruitment of factors that mediate this compaction and concomitant gene silencing[108]. Consistent with the activating role of H3K9ac in model organisms, this mark is involved in flagging *P. falciparum* housekeeping genes[109]. Interestingly, mutually exclusive occurrence of H3K9me3 and H3K9ac was also demonstrated at the level of individual *var* genes. Upon activation, *var* loci are devoid of H3K9me3 and characterised by N-acetylated lysine residues instead[95,106,107]. Whereas H3K9me3 is found at the entire locus of silenced *var* genes, H3K9ac predominantly occupies the active *var ups* region that, during transcription, is additionally marked by di- and tri-methylated H3K4. One of these methylation marks, H3K4me2, bookmarks the active gene during the transcriptionally inactive trophozoite and schizont stages and thus allows for transgenerational inheritance of the *var* gene expression profile[106]. Recent evidence suggests that PfSET10, a H3K4-specific methyltransferase expressed during the second half of the IDC, is involved in this process. Intriguingly, the enzyme accumulates within a unique perinuclear compartment where it co-localises with the poised *var2csa* locus, but is not associated with the inactive form of this *var* gene[110]. PfSET10 thus likely marks the active gene within the *var* expression site.

Given the mutually exclusive occurrence of acetylation and methylation marks at H3K9 it is not surprising that *P. falciparum* sirtuin-like histone deacetylases SIR2A and SIR2B are directly involved in *var* gene silencing and singular gene choice. While PfSIR2A specifically deacetylates H3K9 and H3K14, as well as lysine at position 16 of H4[111], the putative

mammalian counterpart of PfSIR2B was shown to act on acetylation marks of H3K9 and H3K56[112,113]. Transcriptional profiling revealed that deletion of *Pfsir2a* induces up-regulation of a subgroup of *var* genes, particularly affecting members controlled by *upsA, upsC* and *upsE* regions[114,115]. In line with the mutually exclusive relationship between H3K9me3 and H3K9ac, the lack of PfSIR2A activity in knock-out cells lowers the abundance of repressive H3K9me3 marks[95]. Similar to what was observed for Δ*Pfsir2a* cells, parasites lacking *Pfsir2b* are characterised by a de-repression of *var* genes[114]. By contrast, however, here *B*-type genes were most significantly affected, indicating a complementary function of the two sirtuins in *var* gene silencing.

Interestingly, in either of the *Pfsir2* knock-out lines *var* gene de-repression was accompanied by the enrichment of PfH2A.Z at promoter-proximal nucleosomes of the respective *ups* regions[116]. In model organisms, the alternative H2A.Z histone variant is associated with transcriptionally active promoters as well as with the upstream region of poised genes[117–120]. Recent investigations by Petter *et al*. reinforce the view that *var* gene activation involves the specific and temporally regulated exchange of canonical H2A and H2B with the enhancing PfH2A.Z and PfH2B.Z variants, respectively[121]. Together, these findings add another layer of complexity to the epigenetic mechanisms that control singular *var* gene choice.

1.4.3. Heterochromatin Protein 1

HP1 is a conserved protein present in eukaryotes from fission yeast to mammals and is consistently involved in heterochromatin formation and gene silencing. The N-terminal chromodomain (CD) of HP1 binds specifically to H3K9me2/3 and the C-terminal chromoshadow domain (CSD) mediates both homo- and hetero-dimerisation[122]. Importantly, HP1 recruits a H3K9-specific methyltransferase that provides neighbouring nucleosomes with new methylation marks[123–125]. Therefore, heterochromatin is able to spread over nucleosomal arrays in a self-perpetuating manner[126]. Beyond gene silencing, HP1 members are involved in various critical aspects of the nucleus. These include roles in genome function and stability such as sister chromatid cohesion, telomere maintenance, and DNA replication and repair; but HP1 also fulfils tasks at actively transcribed genes[127–130]. At least partially, this functional diversity can be attributed to different isoforms of the protein. In contrast to various other species, however, *P. falciparum* encodes a single HP1 protein only, and several attempts to generate a knockout parasite line failed, suggesting an essential role of PfHP1 in parasite biology[131]. Genome-wide chromatin immunoprecipitation revealed an extraordinarily biased

localisation of PfHP1 to species-specific virulence gene clusters in both subtelomeric and chromosome internal areas including all *var* gene loci and nearly all members of other gene families coding for exported proteins involved in host-parasite interactions[131]. Besides its association with heterochromatic domains, PfHP1 was also found to occupy a small number of euchromatic sites including one locus that encodes an ApiAP2 factor[131]. Interestingly, large heterochromatic regions appear to accept PfHP1-free islands. Specifically, *var2csa* was found to be associated with PfHP1 only when silenced but not in VAR2CSA expressing parasites, pointing towards a dynamic role of PfHP1 in CVGE[132]. It has been proposed that the activation of all variegated genes is subject to a common regulatory mechanism that involves the reversible and locus specific elimination of H3K9me3 and heterochromatin[133].

1.4.4. PfSETvs – a New Player in *var* Gene Regulation

Recently, Jiang and colleagues demonstrated a prominent role for lysine histone methyltransferase PfSETvs – acting on H3K36 – in *var* gene silencing[134]. The authors showed that active *var* gene transcription is characterised by reduced H3K36me3 levels, particularly at the transcriptional start site and the intron region. Consistent with this finding, the entire *var* gene family was found to be de-repressed in PfSETvs-depleted parasites. It is important to note that up-regulation of *var* gene transcription in these cells was accompanied by the loss of H3K9me3 as well[134]. Hence, methylation at H3K36 and H3K9 appear to be closely linked and it will be interesting to find out more about this functional connection. Similar to *var* mRNA, the production of intron-derived antisense transcripts was up-regulated in Δ*Pfsetvs* cells[134]. The simultaneous induction of *ups* and intron promoter activities in response to reduced H3K36me3 reinforces the view of a regulatory interplay between the two elements. How these recent data exactly fit into the current model of intron-mediated *var* gene silencing and mutual exclusion, however, remains to be defined.

1.4.5. The *var2csa* uORF

Although it is well established that *var* genes are mainly controlled at the level of transcription, post-transcriptional mechanisms have been identified to play an important role in regulating the expression of VAR2CSA. More specifically, translation initiation at an uORF in the *var2csa* 5` UTR was shown to prevent protein expression in a reversible manner[51]. Noteworthy, this phenomenon is independent of production of the uORF-encoded peptide. Recent results strongly suggest that the efficiency at which ribosomes re-initiate at the main ORF is rate limiting for VAR2CSA synthesis[54]. Thus, in analogy to the well-known example of yeast GCN4 regulation[135], translational repression of *var2csa* transcripts may be

reversed by an external trigger that allows parasites to rapidly switch to the CSA-binding phenotype. Noteworthy, *var2csa* is controlled by the unique *upsE* sequence; it may therefore be misleading to extrapolate these results to other *var* genes. In fact, to this day we lack evidence for a further contribution of post-transcriptional mechanisms in *var* gene regulation.

1.5. Objectives

The overall objective of my research was to characterise the dynamic chromatin composition that forms the basis for variegated *var* gene expression in *P. falciparum*. More specifically, I aimed at the identification of regulatory *var* promoter elements and their *trans*-acting interaction partners. These efforts yielded two separate projects. While the first one focused on control layers acting on transcription (chapter 2), the second one addressed the issue of post-transcriptional *var* gene regulation (chapter 3). In a third project, I analysed the functional role of PfHP1 in virulence gene expression (chapter 4).

I am both confident and hopeful that my work will add to our understanding of the epigenetic strategy employed by *P. falciparum* to mediate antigenic variation of PfEMP1 and other virulence factor families.

1.6. References

1. WHO. World Malaria Report 2012 FACT SHEET. (2012).
2. Smith, T. G., Lourenço, P., Carter, R., Walliker, D. & Ranford-Cartwright, L. C. Commitment to sexual differentiation in the human malaria parasite, Plasmodium falciparum. *Parasitology* **121 (Pt 2),** 127–133 (2000).
3. Rowe, J. A., Moulds, J. M., Newbold, C. I. & Miller, L. H. P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature* **388,** 292–295 (1997).
4. Baruch, D. I., Gormely, J. A., Ma, C., Howard, R. J. & Pasloske, B. L. Plasmodium falciparum erythrocyte membrane protein 1 is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 3497–3502 (1996).
5. Reeder, J. C. *et al.* The adhesion of Plasmodium falciparum-infected erythrocytes to chondroitin sulfate A is mediated by P. falciparum erythrocyte membrane protein 1. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 5198–5202 (1999).
6. Kyes, S., Horrocks, P. & Newbold, C. Antigenic variation at the infected red cell surface in malaria. *Annu. Rev. Microbiol.* **55,** 673–707 (2001).
7. Baker, T. A. & Bell, S. P. Polymerases and the replisome: machines within machines. *Cell* **92,** 295–305 (1998).
8. Kornberg, R. D. Eukaryotic transcriptional control. *Trends Cell Biol.* **9,** M46–49 (1999).
9. Kornberg, R. D. The molecular basis of eukaryotic transcription. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 12955–12961 (2007).
10. Horrocks, P., Wong, E., Russell, K. & Emes, R. D. Control of gene expression in Plasmodium falciparum – Ten years on. *Mol. Biochem. Parasitol.* **164,** 9–25 (2009).
11. Kishore, S. P., Perkins, S. L., Templeton, T. J. & Deitsch, K. W. An unusual recent expansion of the C-terminal domain of RNA polymerase II in primate malaria parasites features a motif otherwise found only in mammalian polymerases. *J. Mol. Evol.* **68,** 706–714 (2009).
12. Coulson, R. M. R., Hall, N. & Ouzounis, C. A. Comparative genomics of transcriptional control in the human malaria parasite Plasmodium falciparum. *Genome Res.* **14,** 1548–1554 (2004).
13. Callebaut, I., Prat, K., Meurice, E., Mornon, J.-P. & Tomavo, S. Prediction of the general transcription factors associated with RNA polymerase II in Plasmodium falciparum: conserved features and differences relative to other eukaryotes. *BMC Genomics* **6,** 100 (2005).
14. Bozdech, Z. *et al.* The transcriptome of the intraerythrocytic developmental cycle of Plasmodium falciparum. *PLoS Biol.* **1,** E5 (2003).
15. Llinás, M., Bozdech, Z., Wong, E. D., Adai, A. T. & DeRisi, J. L. Comparative whole genome transcriptome analysis of three Plasmodium falciparum strains. *Nucleic Acids Res.* **34,** 1166–1173 (2006).
16. Otto, T. D. *et al.* New insights into the blood-stage transcriptome of Plasmodium falciparum using RNA-Seq. *Mol. Microbiol.* **76,** 12–24 (2010).
17. Foth, B. J. *et al.* Quantitative time-course profiling of parasite and host cell proteins in the human malaria parasite Plasmodium falciparum. *Mol. Cell. Proteomics MCP* **10,** M110.006411 (2011).
18. Florens, L. *et al.* A proteomic view of the Plasmodium falciparum life cycle. *Nature* **419,** 520–526 (2002).
19. Lasonder, E. *et al.* Analysis of the Plasmodium falciparum proteome by high-accuracy mass spectrometry. *Nature* **419,** 537–542 (2002).

20. Le Roch, K. G. *et al.* Global analysis of transcript and protein levels across the Plasmodium falciparum life cycle. *Genome Res.* **14,** 2308–2318 (2004).
21. Nirmalan, N., Sims, P. F. G. & Hyde, J. E. Quantitative proteomics of the human malaria parasite Plasmodium falciparum and its application to studies of development and inhibition. *Mol. Microbiol.* **52,** 1187–1199 (2004).
22. Gelhaus, C., Fritsch, J., Krause, E. & Leippe, M. Fractionation and identification of proteins by 2-DE and MS: towards a proteomic analysis of Plasmodium falciparum. *Proteomics* **5,** 4213–4222 (2005).
23. Hall, N. *et al.* A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses. *Science* **307,** 82–86 (2005).
24. Tarun, A. S. *et al.* A combined transcriptome and proteome survey of malaria parasite liver stages. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 305–310 (2008).
25. Foth, B. J., Zhang, N., Mok, S., Preiser, P. R. & Bozdech, Z. Quantitative protein expression profiling reveals extensive post-transcriptional regulation and post-translational modifications in schizont-stage malaria parasites. *Genome Biol.* **9,** R177 (2008).
26. Briolant, S. *et al.* Plasmodium falciparum proteome changes in response to doxycycline treatment. *Malar. J.* **9,** 141 (2010).
27. Cortés, A., Crowley, V. M., Vaquero, A. & Voss, T. S. A view on the role of epigenetics in the biology of malaria parasites. *PLoS Pathog.* **8,** e1002943 (2012).
28. Hoeijmakers, W. A. M., Stunnenberg, H. G. & Bártfai, R. Placing the Plasmodium falciparum epigenome on the map. *Trends Parasitol.* **28,** 486–495 (2012).
29. Hakimi, M.-A. & Deitsch, K. W. Epigenetics in Apicomplexa: control of gene expression during cell cycle progression, differentiation and antigenic variation. *Curr. Opin. Microbiol.* **10,** 357–362 (2007).
30. Dzikowski, R. & Deitsch, K. W. Genetics of antigenic variation in Plasmodium falciparum. *Curr. Genet.* **55,** 103–110 (2009).
31. Scherf, A., Lopez-Rubio, J. J. & Riviere, L. Antigenic variation in Plasmodium falciparum. *Annu. Rev. Microbiol.* **62,** 445–470 (2008).
32. Aravind, L., Iyer, L. M., Wellems, T. E. & Miller, L. H. Plasmodium biology: genomic gleanings. *Cell* **115,** 771–785 (2003).
33. Balaji, S., Babu, M. M., Iyer, L. M. & Aravind, L. Discovery of the principal specific transcription factors of Apicomplexa and their implication for the evolution of the AP2-integrase DNA binding domains. *Nucleic Acids Res.* **33,** 3994–4006 (2005).
34. De Silva, E. K. *et al.* Specific DNA-binding by apicomplexan AP2 transcription factors. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 8393–8398 (2008).
35. Campbell, T. L., De Silva, E. K., Olszewski, K. L., Elemento, O. & Llinás, M. Identification and genome-wide prediction of DNA binding specificities for the ApiAP2 family of regulators from the malaria parasite. *PLoS Pathog.* **6,** e1001165 (2010).
36. Yuda, M. *et al.* Identification of a transcription factor in the mosquito-invasive stage of malaria parasites. *Mol. Microbiol.* **71,** 1402–1414 (2009).
37. Yuda, M., Iwanaga, S., Shigenobu, S., Kato, T. & Kaneko, I. Transcription factor AP2-Sp and its target genes in malarial sporozoites. *Mol. Microbiol.* **75,** 854–863 (2010).
38. Iwanaga, S., Kaneko, I., Kato, T. & Yuda, M. Identification of an AP2-family protein that is critical for malaria liver stage development. *PloS One* **7,** e47557 (2012).
39. Le Roch, K. G. *et al.* Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science* **301,** 1503–1508 (2003).
40. Painter, H. J., Campbell, T. L. & Llinás, M. The Apicomplexan AP2 family: integral factors regulating Plasmodium development. *Mol. Biochem. Parasitol.* **176,** 1–7 (2011).
41. Van Noort, V. & Huynen, M. A. Combinatorial gene regulation in Plasmodium falciparum. *Trends Genet. TIG* **22,** 73–78 (2006).

42. Flueck, C. *et al.* A major role for the Plasmodium falciparum ApiAP2 protein PfSIP2 in chromosome end biology. *PLoS Pathog.* **6,** e1000784 (2010).
43. Boschet, C. *et al.* Characterization of PfMyb1 transcription factor during erythrocytic development of 3D7 and F12 Plasmodium falciparum clones. *Mol. Biochem. Parasitol.* **138,** 159–163 (2004).
44. Gissot, M., Briquet, S., Refour, P., Boschet, C. & Vaquero, C. PfMyb1, a Plasmodium falciparum transcription factor, is required for intra-erythrocytic growth and controls key genes for cell cycle regulation. *J. Mol. Biol.* **346,** 29–42 (2005).
45. Komaki-Yasuda, K., Okuwaki, M., Nagata, K., Kawazu, S.-I. & Kano, S. Identification of a Novel and Unique Transcription Factor in the Intraerythrocytic Stage of Plasmodium falciparum. *PloS One* **8,** e74701 (2013).
46. Shock, J. L., Fischer, K. F. & DeRisi, J. L. Whole-genome analysis of mRNA decay in Plasmodium falciparum reveals a global lengthening of mRNA half-life during the intra-erythrocytic development cycle. *Genome Biol.* **8,** R134 (2007).
47. Lai, W. S., Carballo, E., Thorn, J. M., Kennington, E. A. & Blackshear, P. J. Interactions of CCCH zinc finger proteins with mRNA. Binding of tristetraprolin-related zinc finger proteins to Au-rich elements and destabilization of mRNA. *J. Biol. Chem.* **275,** 17827–17837 (2000).
48. Braks, J. A. M., Mair, G. R., Franke-Fayard, B., Janse, C. J. & Waters, A. P. A conserved U-rich RNA region implicated in regulation of translation in Plasmodium female gametocytes. *Nucleic Acids Res.* **36,** 1176–1186 (2008).
49. Zhang, M. *et al.* The Plasmodium eukaryotic initiation factor-2alpha kinase IK2 controls the latency of sporozoites in the mosquito salivary glands. *J. Exp. Med.* **207,** 1465–1474 (2010).
50. Epp, C., Li, F., Howitt, C. A., Chookajorn, T. & Deitsch, K. W. Chromatin associated sense and antisense noncoding RNAs are transcribed from the var gene family of virulence genes of the malaria parasite Plasmodium falciparum. *RNA New York N* **15,** 116–127 (2009).
51. Amulic, B., Salanti, A., Lavstsen, T., Nielsen, M. A. & Deitsch, K. W. An upstream open reading frame controls translation of var2csa, a gene implicated in placental malaria. *PLoS Pathog.* **5,** e1000256 (2009).
52. Broadbent, K. M. *et al.* A global transcriptional analysis of Plasmodium falciparum malaria reveals a novel family of telomere-associated lncRNAs. *Genome Biol.* **12,** R56 (2011).
53. Sierra-Miranda, M. *et al.* Two long non-coding RNAs generated from subtelomeric regions accumulate in a novel perinuclear compartment in Plasmodium falciparum. *Mol. Biochem. Parasitol.* **185,** 36–47 (2012).
54. Bancells, C. & Deitsch, K. W. A molecular switch in the efficiency of translation reinitiation controls expression of var2csa, a gene implicated in pregnancy associated malaria. *Mol. Microbiol.* (2013). doi:10.1111/mmi.12379
55. Cooke, B. M., Mohandas, N. & Coppel, R. L. The malaria-infected red blood cell: structural and functional changes. *Adv. Parasitol.* **50,** 1–86 (2001).
56. Biggs, B. A. *et al.* Antigenic variation in Plasmodium falciparum. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 9171–9174 (1991).
57. Magowan, C., Wollish, W., Anderson, L. & Leech, J. Cytoadherence by Plasmodium falciparum-infected erythrocytes is correlated with the expression of a family of variable proteins on infected erythrocytes. *J. Exp. Med.* **168,** 1307–1320 (1988).
58. Ockenhouse, C. F., Magowan, C. & Chulay, J. D. Activation of monocytes and platelets by monoclonal antibodies or malaria-infected erythrocytes binding to the CD36 surface receptor in vitro. *J. Clin. Invest.* **84,** 468–475 (1989).

59. Urban, B. C. *et al.* Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells. *Nature* **400,** 73–77 (1999).
60. Elliott, S. R. *et al.* Inhibition of dendritic cell maturation by malaria is dose dependent and does not require Plasmodium falciparum erythrocyte membrane protein 1. *Infect. Immun.* **75,** 3621–3632 (2007).
61. Kraemer, S. M. & Smith, J. D. A family affair: var genes, PfEMP1 binding, and malaria disease. *Curr. Opin. Microbiol.* **9,** 374–380 (2006).
62. Miller, L. H., Baruch, D. I., Marsh, K. & Doumbo, O. K. The pathogenic basis of malaria. *Nature* **415,** 673–679 (2002).
63. Robinson, B. A., Welch, T. L. & Smith, J. D. Widespread functional specialization of Plasmodium falciparum erythrocyte membrane protein 1 family members to bind CD36 analysed across a parasite genome. *Mol. Microbiol.* **47,** 1265–1278 (2003).
64. Warrell, D. A. Cerebral malaria: clinical features, pathophysiology and treatment. *Ann. Trop. Med. Parasitol.* **91,** 875–884 (1997).
65. MacPherson, G. G., Warrell, M. J., White, N. J., Looareesuwan, S. & Warrell, D. A. Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *Am. J. Pathol.* **119,** 385–401 (1985).
66. Fried, M. & Duffy, P. E. Adherence of Plasmodium falciparum to chondroitin sulfate A in the human placenta. *Science* **272,** 1502–1504 (1996).
67. Salanti, A. *et al.* Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. *J. Exp. Med.* **200,** 1197–1203 (2004).
68. Gardner, M. J. *et al.* Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature* **419,** 498–511 (2002).
69. Su, X. Z. *et al.* The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. *Cell* **82,** 89–100 (1995).
70. Hernandez-Rivas, R. *et al.* Expressed var genes are found in Plasmodium falciparum subtelomeric regions. *Mol. Cell. Biol.* **17,** 604–611 (1997).
71. Rubio, J. P., Thompson, J. K. & Cowman, A. F. The var genes of Plasmodium falciparum are located in the subtelomeric region of most chromosomes. *EMBO J.* **15,** 4069–4077 (1996).
72. Freitas-Junior, L. H. *et al.* Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of P. falciparum. *Nature* **407,** 1018–1022 (2000).
73. Baruch, D. I. *et al.* Cloning the P. falciparum gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. *Cell* **82,** 77–87 (1995).
74. Smith, J. D., Subramanian, G., Gamain, B., Baruch, D. I. & Miller, L. H. Classification of adhesive domains in the Plasmodium falciparum erythrocyte membrane protein 1 family. *Mol. Biochem. Parasitol.* **110,** 293–310 (2000).
75. Kilejian, A., Rashid, M. A., Aikawa, M., Aji, T. & Yang, Y. F. Selective association of a fragment of the knob protein with spectrin, actin and the red cell membrane. *Mol. Biochem. Parasitol.* **44,** 175–181 (1991).
76. Oh, S. S. *et al.* Plasmodium falciparum erythrocyte membrane protein 1 is anchored to the actin-spectrin junction and knob-associated histidine-rich protein in the erythrocyte skeleton. *Mol. Biochem. Parasitol.* **108,** 237–247 (2000).
77. Pei, X. *et al.* Structural and functional studies of interaction between Plasmodium falciparum knob-associated histidine-rich protein (KAHRP) and erythrocyte spectrin. *J. Biol. Chem.* **280,** 31166–31171 (2005).
78. Kyes, S. A., Kraemer, S. M. & Smith, J. D. Antigenic variation in Plasmodium falciparum: gene organization and regulation of the var multigene family. *Eukaryot. Cell* **6,** 1511–1520 (2007).

79. Lavstsen, T., Salanti, A., Jensen, A. T. R., Arnot, D. E. & Theander, T. G. Sub-grouping of Plasmodium falciparum 3D7 var genes based on sequence analysis of coding and non-coding regions. *Malar. J.* **2,** 27 (2003).
80. Kyes, S. A. *et al.* A well-conserved Plasmodium falciparum var gene shows an unusual stage-specific transcript pattern. *Mol. Microbiol.* **48,** 1339–1348 (2003).
81. Scherf, A. *et al.* Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum. *EMBO J.* **17,** 5418–5426 (1998).
82. Kyes, S. *et al.* Plasmodium falciparum var gene expression is developmentally controlled at the level of RNA polymerase II-mediated transcription initiation. *Mol. Microbiol.* **63,** 1237–1247 (2007).
83. Deitsch, K. W., Calderwood, M. S. & Wellems, T. E. Malaria. Cooperative silencing elements in var genes. *Nature* **412,** 875–876 (2001).
84. Voss, T. S. *et al.* A var gene promoter controls allelic exclusion of virulence genes in Plasmodium falciparum malaria. *Nature* **439,** 1004–1008 (2006).
85. Voss, T. S. *et al.* Alterations in local chromatin environment are involved in silencing and activation of subtelomeric var genes in Plasmodium falciparum. *Mol. Microbiol.* **66,** 139–150 (2007).
86. Calderwood, M. S., Gannoun-Zaki, L., Wellems, T. E. & Deitsch, K. W. Plasmodium falciparum var genes are regulated by two regions with separate promoters, one upstream of the coding region and a second within the intron. *J. Biol. Chem.* **278,** 34125–34132 (2003).
87. Gannoun-Zaki, L., Jost, A., Mu, J., Deitsch, K. W. & Wellems, T. E. A silenced Plasmodium falciparum var promoter can be activated in vivo through spontaneous deletion of a silencing element in the intron. *Eukaryot. Cell* **4,** 490–492 (2005).
88. Dzikowski, R., Frank, M. & Deitsch, K. Mutually exclusive expression of virulence genes by malaria parasites is regulated independently of antigen production. *PLoS Pathog.* **2,** e22 (2006).
89. Guizetti, J. & Scherf, A. Silence, activate, poise and switch! Mechanisms of antigenic variation in Plasmodium falciparum. *Cell. Microbiol.* **15,** 718–726 (2013).
90. Marty, A. J. *et al.* Evidence that Plasmodium falciparum chromosome end clusters are cross-linked by protein and are the sites of both virulence gene silencing and activation. *Mol. Microbiol.* **62,** 72–83 (2006).
91. Andrulis, E. D., Neiman, A. M., Zappulla, D. C. & Sternglanz, R. Perinuclear localization of chromatin facilitates transcriptional silencing. *Nature* **394,** 592–595 (1998).
92. Ralph, S. A., Scheidig-Benatar, C. & Scherf, A. Antigenic variation in Plasmodium falciparum is associated with movement of var loci between subnuclear locations. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 5414–5419 (2005).
93. Duraisingh, M. T. *et al.* Heterochromatin silencing and locus repositioning linked to regulation of virulence genes in Plasmodium falciparum. *Cell* **121,** 13–24 (2005).
94. Freitas-Junior, L. H. *et al.* Telomeric heterochromatin propagation and histone acetylation control mutually exclusive expression of antigenic variation genes in malaria parasites. *Cell* **121,** 25–36 (2005).
95. Lopez-Rubio, J.-J., Mancio-Silva, L. & Scherf, A. Genome-wide analysis of heterochromatin associates clonally variant gene regulation with perinuclear repressive centers in malaria parasites. *Cell Host Microbe* **5,** 179–190 (2009).
96. Dzikowski, R. *et al.* Mechanisms underlying mutually exclusive expression of virulence genes by malaria parasites. *EMBO Rep.* **8,** 959–965 (2007).

97. Mok, B. W. *et al.* Comparative transcriptomal analysis of isogenic Plasmodium falciparum clones of distinct antigenic and adhesive phenotypes. *Mol. Biochem. Parasitol.* **151,** 184–192 (2007).
98. Niang, M., Yan Yam, X. & Preiser, P. R. The Plasmodium falciparum STEVOR multigene family mediates antigenic variation of the infected erythrocyte. *PLoS Pathog.* **5,** e1000307 (2009).
99. Lavazec, C., Sanyal, S. & Templeton, T. J. Expression switching in the stevor and Pfmc-2TM superfamilies in Plasmodium falciparum. *Mol. Microbiol.* **64,** 1621–1634 (2007).
100. Blythe, J. E. *et al.* Plasmodium falciparum STEVOR proteins are highly expressed in patient isolates and located in the surface membranes of infected red blood cells and the apical tips of merozoites. *Infect. Immun.* **76,** 3329–3336 (2008).
101. Fernandez, V., Hommel, M., Chen, Q., Hagblom, P. & Wahlgren, M. Small, clonally variant antigens expressed on the surface of the Plasmodium falciparum-infected erythrocyte are encoded by the rif gene family and are the target of human immune responses. *J. Exp. Med.* **190,** 1393–1404 (1999).
102. Witmer, K. *et al.* Analysis of subtelomeric virulence gene families in Plasmodium falciparum by comparative transcriptional profiling. *Mol. Microbiol.* **84,** 243–259 (2012).
103. Frank, M. *et al.* Strict pairing of var promoters and introns is required for var gene silencing in the malaria parasite Plasmodium falciparum. *J. Biol. Chem.* **281,** 9942–9952 (2006).
104. Voss, T. S., Kaestli, M., Vogel, D., Bopp, S. & Beck, H.-P. Identification of nuclear proteins that interact differentially with Plasmodium falciparum var gene promoters. *Mol. Microbiol.* **48,** 1593–1607 (2003).
105. Swamy, L., Amulic, B. & Deitsch, K. W. Plasmodium falciparum var gene silencing is determined by cis DNA elements that form stable and heritable interactions. *Eukaryot. Cell* **10,** 530–539 (2011).
106. Lopez-Rubio, J. J. *et al.* 5' flanking region of var genes nucleate histone modification patterns linked to phenotypic inheritance of virulence traits in malaria parasites. *Mol. Microbiol.* **66,** 1296–1305 (2007).
107. Chookajorn, T. *et al.* Epigenetic memory at malaria virulence genes. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 899–902 (2007).
108. Krauss, V. Glimpses of evolution: heterochromatic histone H3K9 methyltransferases left its marks behind. *Genetica* **133,** 93–106 (2008).
109. Cui, L. *et al.* PfGCN5-mediated histone H3 acetylation plays a key role in gene expression in Plasmodium falciparum. *Eukaryot. Cell* **6,** 1219–1227 (2007).
110. Volz, J. C. *et al.* PfSET10, a Plasmodium falciparum methyltransferase, maintains the active var gene in a poised state during parasite division. *Cell Host Microbe* **11,** 7–18 (2012).
111. French, J. B., Cen, Y. & Sauve, A. A. Plasmodium falciparum Sir2 is an NAD+-dependent deacetylase and an acetyllysine-dependent and acetyllysine-independent NAD+ glycohydrolase. *Biochemistry (Mosc.)* **47,** 10227–10239 (2008).
112. Michishita, E. *et al.* Cell cycle-dependent deacetylation of telomeric histone H3 lysine K56 by human SIRT6. *Cell Cycle Georget. Tex* **8,** 2664–2666 (2009).
113. Yang, B., Zwaans, B. M. M., Eckersdorff, M. & Lombard, D. B. The sirtuin SIRT6 deacetylates H3 K56Ac in vivo to promote genomic stability. *Cell Cycle Georget. Tex* **8,** 2662–2663 (2009).
114. Tonkin, C. J. *et al.* Sir2 paralogues cooperate to regulate virulence genes and antigenic variation in Plasmodium falciparum. *PLoS Biol.* **7,** e84 (2009).

115. Merrick, C. J. *et al.* The effect of Plasmodium falciparum Sir2a histone deacetylase on clonal and longitudinal variation in expression of the var family of virulence genes. *Int. J. Parasitol.* **40,** 35–43 (2010).
116. Petter, M. *et al.* Expression of P. falciparum var genes involves exchange of the histone variant H2A.Z at the promoter. *PLoS Pathog.* **7,** e1001292 (2011).
117. Barski, A. *et al.* High-resolution profiling of histone methylations in the human genome. *Cell* **129,** 823–837 (2007).
118. Raisner, R. M. *et al.* Histone variant H2A.Z marks the 5' ends of both active and inactive genes in euchromatin. *Cell* **123,** 233–248 (2005).
119. Dalmasso, M. C., Onyango, D. O., Naguleswaran, A., Sullivan, W. J., Jr & Angel, S. O. Toxoplasma H2A variants reveal novel insights into nucleosome composition and functions for this histone family. *J. Mol. Biol.* **392,** 33–47 (2009).
120. Zhang, H., Roberts, D. N. & Cairns, B. R. Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss. *Cell* **123,** 219–231 (2005).
121. Petter, M. *et al.* H2A.Z and H2B.Z double-variant nucleosomes define intergenic regions and dynamically occupy var gene promoters in the malaria parasite Plasmodium falciparum. *Mol. Microbiol.* **87,** 1167–1182 (2013).
122. Lomberk, G., Wallrath, L. & Urrutia, R. The Heterochromatin Protein 1 family. *Genome Biol.* **7,** 228 (2006).
123. Aagaard, L. *et al.* Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31. *EMBO J.* **18,** 1923–1938 (1999).
124. Schotta, G. *et al.* Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. *EMBO J.* **21,** 1121–1131 (2002).
125. Fritsch, L. *et al.* A subset of the histone H3 lysine 9 methyltransferases Suv39h1, G9a, GLP, and SETDB1 participate in a multimeric complex. *Mol. Cell* **37,** 46–56 (2010).
126. Li, Y., Kirschmann, D. A. & Wallrath, L. L. Does heterochromatin protein 1 always follow code? *Proc. Natl. Acad. Sci. U. S. A.* **99 Suppl 4,** 16462–16469 (2002).
127. Kwon, S. H. & Workman, J. L. The heterochromatin protein 1 (HP1) family: put away a bias toward HP1. *Mol. Cells* **26,** 217–227 (2008).
128. Hiragami, K. & Festenstein, R. Heterochromatin protein 1: a pervasive controlling influence. *Cell. Mol. Life Sci. CMLS* **62,** 2711–2726 (2005).
129. Hediger, F. & Gasser, S. M. Heterochromatin protein 1: don't judge the book by its cover! *Curr. Opin. Genet. Dev.* **16,** 143–150 (2006).
130. Zeng, W., Ball, A. R., Jr & Yokomori, K. HP1: heterochromatin binding proteins working the genome. *Epigenetics Off. J. DNA Methylation Soc.* **5,** 287–292 (2010).
131. Flueck, C. *et al.* Plasmodium falciparum heterochromatin protein 1 marks genomic loci linked to phenotypic variation of exported virulence factors. *PLoS Pathog.* **5,** e1000569 (2009).
132. Pérez-Toledo, K. *et al.* Plasmodium falciparum heterochromatin protein 1 binds to tri-methylated histone 3 lysine 9 and is linked to mutually exclusive expression of var genes. *Nucleic Acids Res.* **37,** 2596–2606 (2009).
133. Rovira-Graells, N. *et al.* Transcriptional variation in the malaria parasite Plasmodium falciparum. *Genome Res.* **22,** 925–938 (2012).
134. Jiang, L. *et al.* PfSETvs methylation of histone H3K36 represses virulence genes in Plasmodium falciparum. *Nature* **499,** 223–227 (2013).
135. Mueller, P. P. & Hinnebusch, A. G. Multiple upstream AUG codons mediate translational control of GCN4. *Cell* **45,** 201–207 (1986).

Chapter 2

Identification of a *cis*-acting DNA-protein interaction implicated in singular *var* gene choice in *Plasmodium falciparum*

Nicolas M. B. Brancucci[1,2,#], Kathrin Witmer[1,2,#], Christoph D. Schmid[1,2], Christian Flueck[1,2], and Till S. Voss[1,2,*]

[1]Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, 4051 Basel, Switzerland; [2]University of Basel, 4003 Basel, Switzerland

[#]These authors contributed equally to this work.

*Corresponding author: till.voss@unibas.ch; Phone: +41 61 284 81 61

published

Cellular Microbiology, 2012 Dec;14(12):1836-48. doi: 10.1111/cmi.12004. Epub 2012 Sep 4.

2.1. Abstract

Plasmodium falciparum is responsible for the most severe form of malaria in humans. Antigenic variation of *P. falciparum* erythrocyte membrane protein 1 leads to immune evasion and occurs through switches in mutually exclusive *var* gene transcription. The recent progress in *Plasmodium* epigenetics notwithstanding, the mechanisms by which singularity of *var* activation is achieved are unknown. Here, we employed a functional approach to dissect the role of *var* gene upstream regions in mutually exclusive activation. Besides identifying sequence elements involved in activation and initiation of transcription, we mapped a region downstream of the transcriptional start site that is required to maintain singular *var* gene choice. Activation of promoters lacking this sequence occurs no longer in competition with endogenous *var* genes. Within this region we pinpointed a sequence-specific DNA-protein interaction involving a *cis*-acting sequence motif that is conserved in the majority of *var* loci. These results suggest an important role for this interaction in mutually exclusive locus recognition. Our findings are furthermore consistent with a novel mechanism for the control of singular gene choice in eukaryotes. In addition to their importance in *P. falciparum* antigenic variation, our results may also help to explain similar processes in other systems.

2.2. Introduction

Many unicellular pathogens use antigenic variation to escape adaptive immune responses in the host. The widespread occurrence of this strategy in evolutionary distant species underscores its key role in pathogen survival and spreading. While the underlying control pathways are highly diverse in different systems, both mechanistically and in terms of complexity, antigenic variation is defined by two basic concepts. First, the antigens are encoded by gene families, the members of which are expressed in a mutually exclusive manner. Second, switches in the expression of individual members lead to antigenic variation of surface-exposed antigens. In several medically important pathogens such as *Borrelia* spp., *Neisseria* spp., *Giardia lamblia*, *Plasmodium falciparum* and *Trypanosoma brucei*, this paradigm of clonal phenotypic variation reaches a remarkable yet poorly understood level of sophistication (Deitsch *et al.*, 2009;Dzikowski and Deitsch, 2009;Morrison *et al.*, 2009;Prucca and Lujan, 2009).

The apicomplexan parasite *P. falciparum* causes several hundred million malaria cases and close to one million deaths annually (World Health Organisation, 2010). Malaria-

associated morbidity and mortality is a result of the intra-erythrocytic developmental cycle (IDC) where repeated rounds of parasite invasion into red blood cells (RBCs) are followed by intra-cellular maturation and replication. During this stage of infection parasites expose the major virulence factor *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) on the RBC surface (Leech *et al.*, 1984). This highly polymorphic antigen, encoded by the 60-member *var* gene family, undergoes antigenic variation to facilitate chronic infection and transmission (Biggs *et al.*, 1991;Gardner *et al.*, 2002;Roberts *et al.*, 1992;Smith *et al.*, 1995;Su *et al.*, 1995). Furthermore, PfEMP1 mediates sequestration of infected RBC aggregates in the microvasculature of various organs and is thus directly responsible for severe outcomes, including cerebral and placental malaria (Baruch *et al.*, 1996;Beeson and Duffy, 2005;Gardner *et al.*, 1996;MacPherson *et al.*, 1985;Pongponratn *et al.*, 1991;Reeder *et al.*, 1999).

var genes are transcribed by RNA polymerase II (RNA polII) in ring stage parasites during the first half of the IDC (Dzikowski *et al.*, 2006;Kyes *et al.*, 2007;Scherf *et al.*, 1998). Notably, only one *var* gene is transcribed at any time while all other members are silenced (Scherf *et al.*, 1998). Switches in *var* gene transcription, and consequently antigenic variation of PfEMP1, are independent of detectable recombination events and occur by *in situ var* gene activation (Scherf *et al.*, 1998). *var* gene silencing is explained by the fact that all *var* genes are positioned in subtelomeric and some chromosome-internal heterochromatic regions (Flueck *et al.*, 2009;Gardner *et al.*, 2002;Lopez-Rubio *et al.*, 2009;Salcedo-Amaya *et al.*, 2009). These chromosomal domains are uniformly enriched in histone 3 lysine 9 tri-methylation (H3K9me3) and *P. falciparum* heterochromatin protein 1 (PfHP1) (Flueck *et al.*, 2009;Lopez-Rubio *et al.*, 2009;Perez-Toledo *et al.*, 2009;Salcedo-Amaya *et al.*, 2009). The presence of these epigenetic marks is directly linked to *var* gene silencing (Chookajorn *et al.*, 2007;Lopez-Rubio *et al.*, 2007;Perez-Toledo *et al.*, 2009). In contrast, the active *var* locus is associated with H3K9ac and H3K4me2/me3 instead (Lopez-Rubio *et al.*, 2007). Interestingly, singular *var* gene activation is linked to locus repositioning into a dedicated perinuclear expression site (Duraisingh *et al.*, 2005;Dzikowski *et al.*, 2007;Marty *et al.*, 2006;Ralph *et al.*, 2005;Voss *et al.*, 2006). While the mechanisms underlying this process are largely unknown, a recent study identified a critical role for nuclear actin in locus repositioning and mutually exclusive expression (Zhang *et al.*, 2011). Moreover, Volz et al. identified a H3K4-specific methyltransferase (PfSET10) and demonstrated its exclusive localisation to the active *var* locus suggesting a role for this enzyme in the transmission of epigenetic memory (Volz *et al.*, 2012).

In recent years, *var* gene promoters emerged as key components in all layers of *var* gene regulation. Experiments where *var* gene promoters drive transcription of drug-selectable reporter genes have been particularly informative in studying *var* promoter function. In absence of drug selection *var* promoters are predominantly silenced, whereas drug challenge selects for parasites carrying active promoters (Voss *et al.*, 2006;Voss *et al.*, 2007). Importantly, this forced activation is sufficient to infiltrate a drug-selectable reporter into the mutual exclusion programme (Dzikowski *et al.*, 2007;Voss *et al.*, 2006;Voss *et al.*, 2007). In addition to *var* promoters, the *var* intron acts as a cooperative partner in silencing and mutual exclusion (Calderwood *et al.*, 2003;Deitsch *et al.*, 2001;Dzikowski *et al.*, 2007;Frank *et al.*, 2006;Gannoun-Zaki *et al.*, 2005;Voss *et al.*, 2006).

We postulated that transcriptional control of *var* genes may be mediated by unknown sequence information contained within the promoter region. In this study, we developed a functional promoter mapping approach tailored to identify and characterise *var* gene-specific regulatory information. We mapped an autonomous upstream activating sequence (UAS) that is essential for *var* promoter activation. Notably, we also identified a region downstream of the transcriptional start site (TSS) and demonstrate an important role for this element in mutually exclusive promoter recognition. In absence of this sequence *var* promoters are fully active but, unlike wild-type promoters, do not compete with endogenous *var* gene transcription. Within this region we identified a 47bp motif that interacts in a sequence-specific manner with an unknown nuclear protein. Together, our results show for the first time that the complex regulation of mutually exclusive *var* gene transcription involves functional *cis*-acting modules with intrinsic and position-dependent activities. They are furthermore consistent with a novel mechanism in sustaining singular gene choice in eukaryotes.

2.3. Results

2.3.1. Functional *var* promoter mapping by bi-directional deletion analysis

To identify regulatory *var* promoter elements we employed a system suitable to analyse promoter activity in stably transfected parasites. All reporter constructs are based on the parental plasmid pBC (Fig. 1A) where the blasticidin deaminase (*bsd*) resistance cassette selects for stable episomes. A 2.5kb *var upsC* upstream sequence (PFL1960w) controls transcription of the dual reporter encoding human dihydrofolate reductase fused to green fluorescent protein (h*dhfr-gfp)*. A *var* gene intron element is located downstream of the

h*dhfr-gfp* cassette to account for its role in *var* gene regulation. A telomere-associated repeat element 6 sequence (TARE6/rep20) is included for improved plasmid segregation (O'Donnell *et al.*, 2002). In such a context, homogenous populations carrying active *upsC* promoters are obtained via selection with the antifolate drug WR99210 (WR) (Voss *et al.*, 2006;Voss *et al.*, 2007).

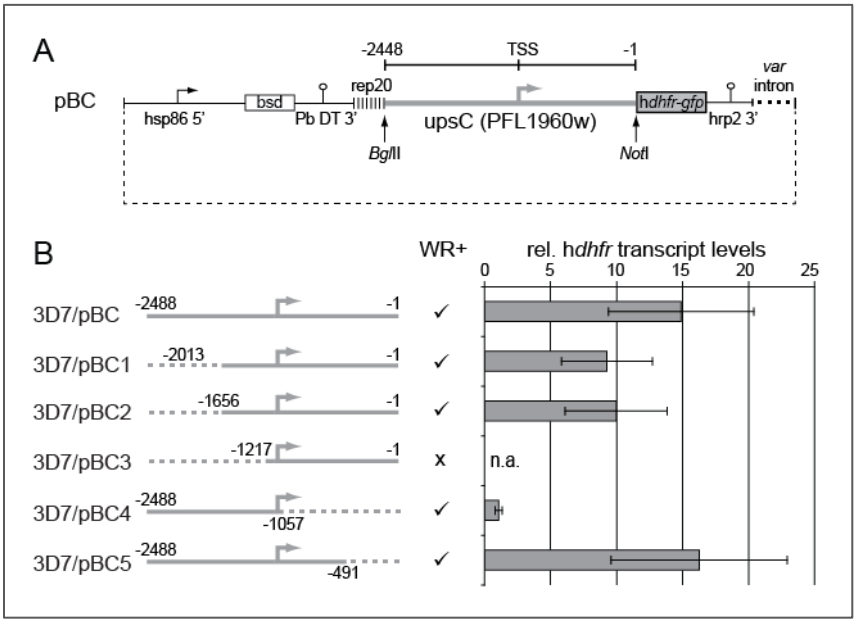


Fig. 1. Functional *var* promoter mapping by serial deletion analysis.
A. Schematic map of pBC. The PFL1960w *upsC* upstream sequence controls transcription of h*dhfr-gfp*. The approximate position of the TSS is indicated (Deitsch *et al.*, 1999). The *bsd* resistance cassette selects for stably transfected parasites. The *var* intron is indicated by a bold dashed line. pBC descendants were obtained by replacing the *upsC* promoter with truncated sequences using *Bgl*II and *Not*I. hsp86 5', *hsp86* promoter; Pb DT 3', *P. berghei dhfr*-thymidylate synthase terminator; rep20, 0.5kb TARE6 repeat element; hrp2 3'; histidine-rich protein 2 terminator.
B. Activities of full-length and truncated promoters in WR-selected parasites. Deletions are represented by dashed lines. Numbers represent nucleotide positions in relation to the ATG. Successful WR-selection is indicated by check marks. Values represent relative h*dhfr-gfp* transcripts normalised against transcription of PF13_0170 (glutaminyl-tRNA synthetase, putative) and plasmid copy number. Values represent the average of three independent experiments (two replicates for 3D7/pBC1 and 3D7/pBC2) (mean +/- SEM). n.a., not applicable.

To identify elements involved in promoter activation and mutual exclusion we sequentially truncated the *upsC* upstream sequence from either the 5' or 3' end (Fig. 1B). We chose this bi-directional approach to identify possible functional regions both up- and downstream of the putative TSS. Based on a multiple *upsC* sequence alignment and the previous experimental mapping of an *upsC* TSS we expected the TSS of PFL1960w at position -1167 (Deitsch *et al.*, 1999;Voss *et al.*, 2000). Transfected parasites were challenged with WR and resistant populations were obtained for all but one cell line, 3D7/pBC3 (Fig.

1B). Several attempts to select for WR-resistant 3D7/pBC3 parasites failed showing that the region between -1656 to -1217 comprises an important UAS and/or the core promoter. To test if any of the deletions affected promoter strength we determined relative h*dhfr-gfp* transcript levels in ring stage parasites by quantitative reverse transcriptase PCR (qRT-PCR). As shown in Fig. 1B, transcript levels in 3D7/pBC1 and 3D7/pBC2 were similar to those in 3D7/pBC indicating that the sequence upstream of -1656 does not contribute to *var* promoter activity. The promoter in pBC5, lacking 491bp of the 5' UTR, was also fully active. In contrast, the truncation encompassing bps -1057 to -1 in pBC4 caused a significant reduction in steady state transcript levels. Hence, this approach identified two regulatory regions, located upstream and downstream of the putative TSS, respectively, which fulfil important roles in *var* promoter function.

2.3.2. Functional identification of an autonomous *upsC* upstream activating sequence

To learn more about the nature of the putative UAS we set out to analyse its function in the context of a minimal heterologous promoter. We decided to use the knob-associated histidine rich protein (*kahrp*) gene promoter for three reasons. Firstly, the TSS of this gene has been mapped to 849bp upstream of the ATG (Lanzer *et al.*, 1992). Secondly, similar to *var* genes the timing of *kahrp* transcription peaks in ring stage parasites. Lastly, the *kahrp* locus is not enriched in H3K9me3/PfHP1 (Flueck *et al.*, 2009;Lopez-Rubio *et al.*, 2009;Salcedo-Amaya *et al.*, 2009), which is an important consideration in order to avoid heterochromatin-mediated masking of autonomous *cis*-acting activities. Hence, we generated plasmid pBK$_{min}$-RI where bps -1115 to -1 of the *kahrp* upstream sequence control transcription of the h*dhfr-gfp* reporter (Fig. 2A). Parasites carrying pBK$_{min}$-RI episomes were readily obtained after transfection. Notably, the disposition of this plasmid to integrate into the endogenous *kahrp* locus allowed us to measure K$_{min}$ activity also in a chromosomal environment. This integration event essentially causes a promoter swap where K$_{min}$ drives expression of the endogenous *kahrp* gene and the endogenous *kahrp* promoter controls transcription of the h*dhfr-gfp* reporter (Figs. 2B and S1). Compared to the endogenous full-length *kahrp* promoter, the episomal and chromosomal minimal promoters displayed a 300-fold and 1000-fold reduced activity, respectively (Fig. 2C). Hence, K$_{min}$ clearly fulfilled the requirements for a minimal promoter. We cloned two overlapping fragments containing the putative *upsC* UAS upstream of K$_{min}$ to create upsC-K$_{min}$ hybrid promoters (pBC1K$_{min}$ and pBC2K$_{min}$) (Figs. 2D and S1).

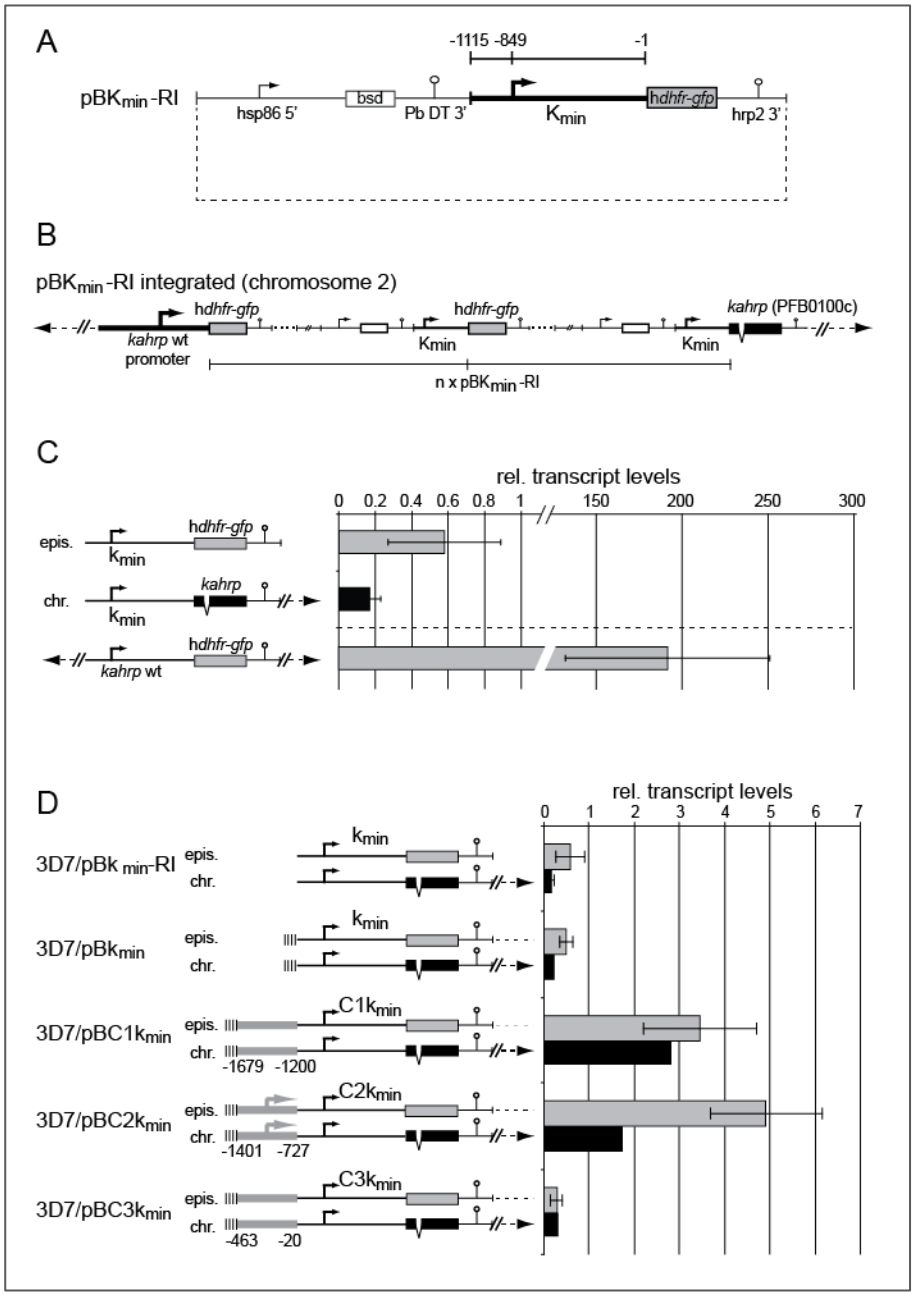


Fig. 2. An *upsC* UAS element activates the minimal promoter K_{min}.

A. Schematic map of pBK$_{min}$-RI. The position of the *kahrp* TSS is indicated (Lanzer *et al.*, 1992).

B. Schematic map of pBK$_{min}$-RI concatamers integrated into the endogenous *kahrp* locus (PFB0100c).

C. Comparison of relative transcript levels produced by the episomal (h*dhfr-gfp* transcripts; grey bar) and chromosomal (*kahrp* transcripts; black bar) K$_{min}$ promoters, and the *kahrp* wild-type promoter (h*dhfr-gfp* transcripts; grey bar) in 3D7/pBK$_{min}$-RI parasites. Values are derived from three independent experiments and represent *msp8*-normalised transcripts (mean +/- SEM). Values for the episomal K$_{min}$ promoter were additionally adjusted for plasmid copy number.

D. Analysis of upsC-K$_{min}$ hybrid promoters. *upsC* insertions are depicted by bold grey lines. The rep20 element is indicated by a vertical array and the *var* intron by a dashed line. The graph compares relative transcript levels (*msp8*-normalised) produced by the episomal (h*dhfr-gfp* transcripts, grey bars) and chromosomal (*kahrp* transcripts, black bars) K$_{min}$ and upsC-K$_{min}$ hybrid promoters. Values for episomal promoters are derived from three independent experiments (mean +/- SEM) and were additionally adjusted for plasmid copy number. Data for 3D7/pBK$_{min}$-RI are identical to those in Figure 2C.

The region downstream of the *upsC* TSS encompassing bps -463 to -20, which has no effect on *upsC* promoter activity (Fig. 1B), was used as negative control (pBC3K_{min}). qRT-PCR analysis revealed that *upsC* fragments C1 (-1679 to -1200) and C2 (-1401 to -727) consistently activated K_{min} to a similar extent in both the episomal and chromosomal context whereas fragment C3 had no effect. Furthermore, neither the *var* intron nor the rep20 element altered K_{min} activity.

Together, these findings corroborate the results obtained with the *upsC* deletion constructs and are consistent with the presence of a *var* UAS located between bps -1401 and -1217. The fact that this element activates transcription from a heterologous minimal promoter suggests an autonomous, context-independent function in activating RNA polII-mediated transcription.

2.3.3. Transcriptional initiation from an alternative TSS compensates for the loss of core promoter function

Here, we investigated the functional region downstream of the putative TSS that is defined by plasmids pBC4 and pBC5 (-1057 to -491). Deletion of this region caused a substantial reduction in steady state transcripts (Fig. 1B), suggesting it may contain important activating sequences. Northern blot analysis confirmed the reduced abundance of steady state transcripts in 3D7/pBC4 compared to 3D7/pBC and 3D7/pBC5 (Fig. 3). An independent time-course experiment confirmed these results and excluded the possibility of altered transcriptional timing and/or transcript accumulation in 3D7/pBC4 parasites (Fig. S2). However, these experiments also revealed that the size difference between pBC- and pBC4-derived transcripts was much smaller than expected. In spite of the 1057bp deletion in the 5' UTR, pBC4-derived transcripts were larger than those originating from pBC5 where only 491bp of the 5' UTR were deleted (Fig. 3). This shows that

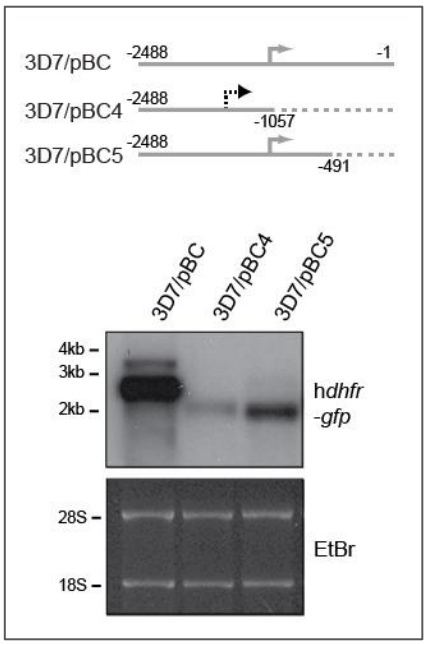


Fig. 3. Transcriptional initiation from an alternative *upsC* upstream TSS.
Identification of an alternative *upsC* upstream TSS (dashed arrow). Full-length and truncated promoters are schematically depicted on top. h*dhfr-gfp* transcript size and abundance was estimated by Northern analysis of total RNA isolated from WR-selected ring stage parasites. Ethidium bromide-stained 18S and 28S rRNAs serve as loading control.

transcription from the truncated pBC4 upstream sequence initiated from an alternative upstream TSS. Consequently, the reduced steady state transcript levels observed in 3D7/pBC4 were not related to the deletion of important activating sequences but rather to the loss of proper core promoter function and transcriptional initiation from the natural TSS.

2.3.4. A regulatory region downstream of the TSS is involved in mutually exclusive *var* gene expression

Transgenic parasites carrying activated full-length *var* promoters do not transcribe endogenous *var* genes and fail to express PfEMP1 (Chookajorn *et al.*, 2007;Dzikowski *et al.*, 2006;Dzikowski *et al.*, 2007;Howitt *et al.*, 2009;Voss *et al.*, 2006;Voss *et al.*, 2007;Witmer *et al.*, 2012). This implies that mutually exclusive locus recognition may be mediated by *cis*-acting regulatory sequence elements located in *var* gene upstream regions. To test this hypothesis and to

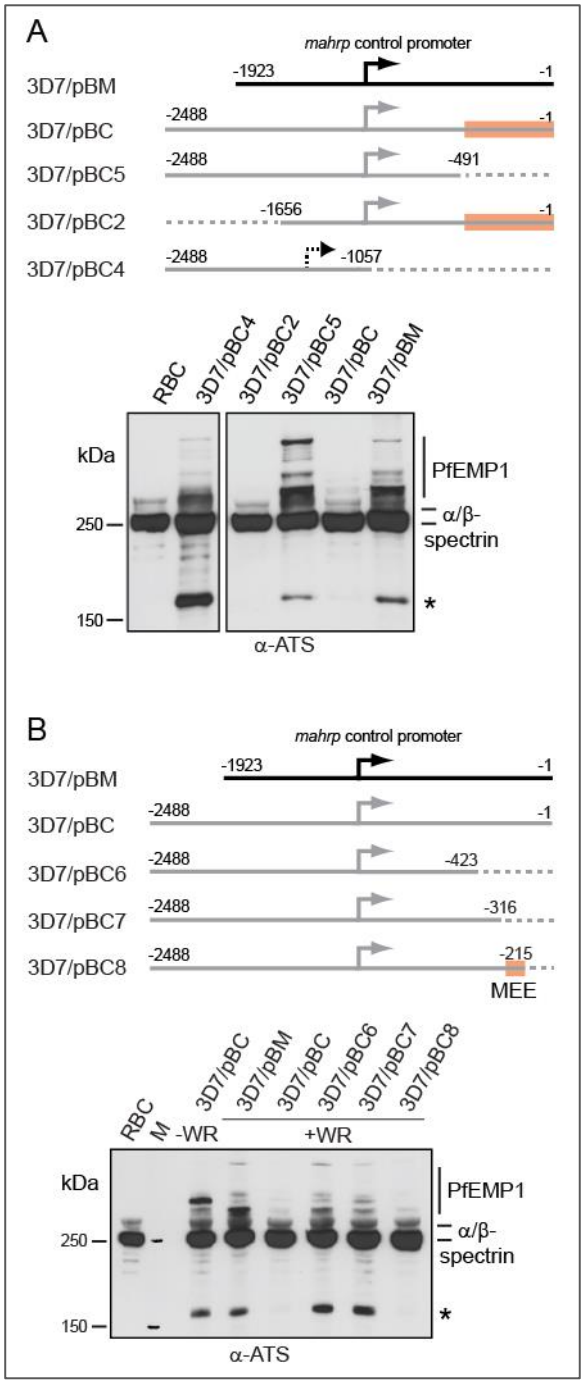


Fig. 4. Mutually exclusive activation is mediated by a 101bp element downstream of the TSS.

A. Functional identification of a mutual exclusion element downstream of the TSS. Promoters are schematically depicted on top. pBM is a negative control construct where the *mahrp1* promoter controls h*dhfr-gfp* transcription. *upsC* sequences are shown in grey. Deletions are represented by dashed lines. The orange box highlights the region required for mutually exclusive activation. PfEMP1 expression in WR-selected trophozoites was monitored by Western blot using antibodies against the conserved ATS domain of PfEMP1 (Duffy *et al.*, 2002). The antibody cross-reacts with human spectrin. PfEMP1 is detected at various sizes above 250kDa. The signal at 160kDa probably represents smaller PfEMP1 species (asterisk). RBC, uninfected RBCs.

B. The mutual exclusion element maps to a 101bp region downstream of the TSS. The orange box identifies the mutual exclusion element (MEE) located at position -316 to -215. WR-selected 3D7/pBM where the *mahrp* promoter controls h*dhfr-gfp* transcription and WR-unselected 3D7/pBC carrying a silenced *upsC* promoter served as negative controls. RBC, uninfected RBCs. M, size standard; -WR, unselected; +WR, WR-selected.

identify such functional elements we investigated if any of the activated truncated promoters escaped mutually exclusive activation. The negative control line 3D7/pBM, in which the unrelated ring stage-specific *mahrp1* promoter controls h*dhfr-gfp* transcription, expressed PfEMP1 at normal levels, whereas parasites of the positive control line 3D7/pBC exhibited the expected PfEMP1 knock-down phenotype (Fig. 4A). PfEMP1 expression was also abolished in 3D7/pBC2 showing that the region ranging from -2488 to -1656 bps upstream of the start codon is not important for mutually exclusive locus recognition. In contrast, 3D7/pBC4 and 3D7/pBC5 parasites expressed PfEMP1 at levels similar to the 3D7/pBM negative control line. Interestingly, both truncated promoters lack the same 491bp sequence downstream of the TSS suggesting that this region carries sequence information important for mutually exclusive locus recognition. To map this region more precisely we cloned three additional truncated *upsC* sequences in pBC6, pBC7 and pBC8 (Fig. 4B). Similar to the full-length promoter in 3D7/pBC, 3D7/pBC8 parasites failed to express PfEMP1 demonstrating that the pBC8 promoter was activated in a mutually exclusive manner. In contrast, 3D7/pBC6 and 3D7/pBC7 expressed PfEMP1 at levels similar to two negative controls (WR-selected 3D7/pBM and unselected 3D7/pBC) showing that these truncated promoters were not subject to mutually exclusive recognition as already observed for 3D7/pBC5. Together, this series of experiments pinpointed a putative 101bp mutual exclusion element (MEE) (bps -316 to -215) that drives the *upsC* promoter into mutually exclusive activation; in absence of the MEE promoters escape this restriction and are activated in parallel to endogenous *var* transcription.

2.3.5. The mutual exclusion element interacts specifically with an unknown nuclear factor

The proposed function of the MEE in mutually exclusive activation may be directly linked to the specific recruitment of an unknown regulatory factor. We therefore tested three overlapping fragments (MEE1 - MEE3) in electromobility shift assays (EMSA) using parasite nuclear extracts. Whereas MEE1 and MEE3 showed no sign of specific binding (data not shown), the central 47bp MEE2 fragment formed a DNA-protein complex that was specifically competed in a dose-dependent manner by an excess of homologous competitor only (Fig. 5A). To characterise this interaction in more detail we performed competition EMSAs using a set of mutated MEE2 sequences (Fig. 5B). As expected, scrambled MEE2 failed to compete – underscoring the sequence-specificity of this interaction. Four out of six fragments carrying consecutively mutated 8mers (MEE2-mut2/-mut3/-mut5/-mut6) competed

with similar efficiency as the MEE2 wild-type sequence (Figs. 5B and S3A). In contrast, MEE2-mut4 failed to compete even at a 100-fold molar excess, and MEE2-mut1 competed with intermediate efficiency. Hence, we conclude that the 8 bp ATAGATTA sequence mutated in MEE2-mut4 represents a core motif necessary for this specific interaction, whereas the 8mer sequence at the 5' end of MEE2 may have ancillary function in complex formation.

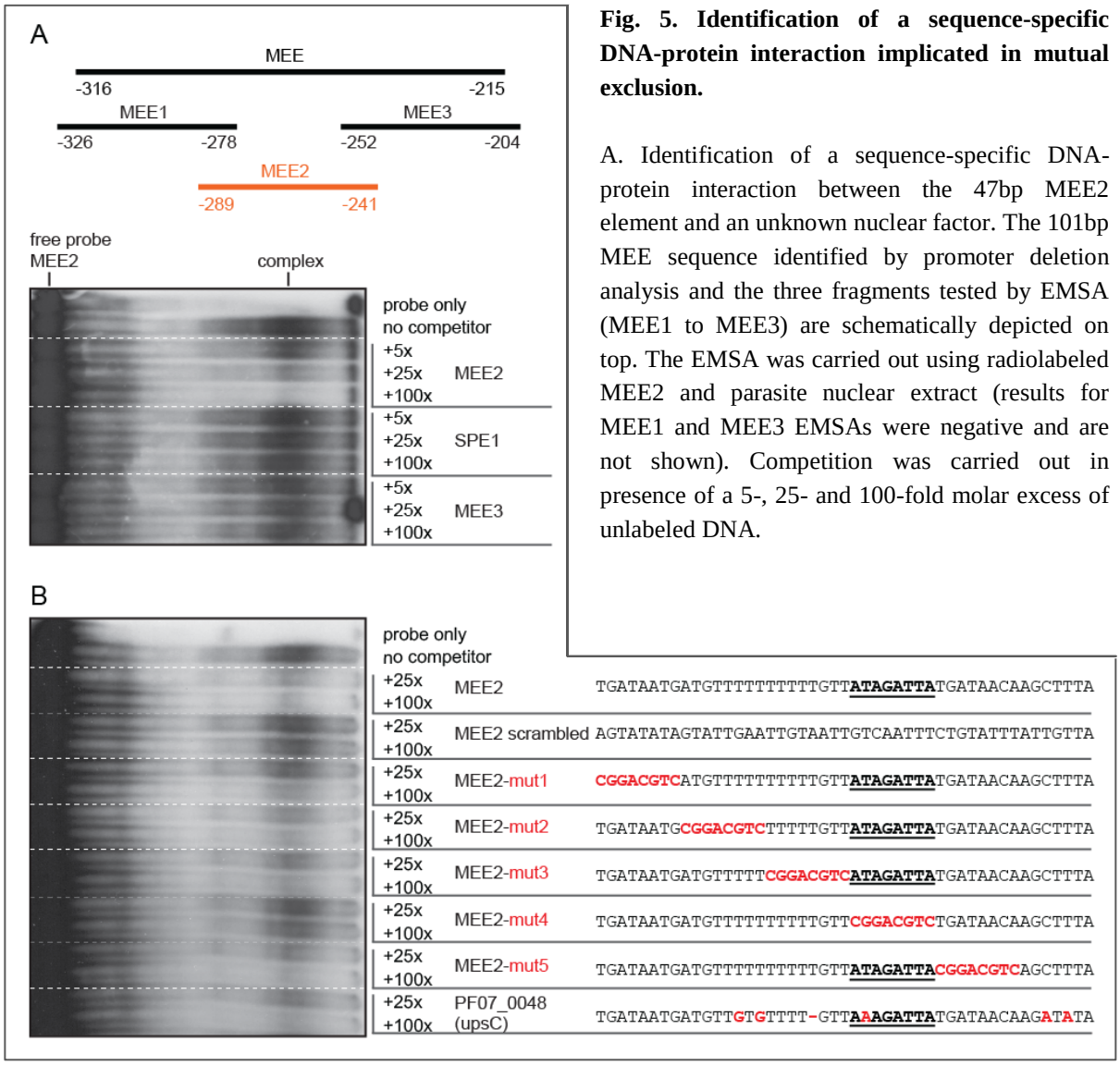


Fig. 5. Identification of a sequence-specific DNA-protein interaction implicated in mutual exclusion.

A. Identification of a sequence-specific DNA-protein interaction between the 47bp MEE2 element and an unknown nuclear factor. The 101bp MEE sequence identified by promoter deletion analysis and the three fragments tested by EMSA (MEE1 to MEE3) are schematically depicted on top. The EMSA was carried out using radiolabeled MEE2 and parasite nuclear extract (results for MEE1 and MEE3 EMSAs were negative and are not shown). Competition was carried out in presence of a 5-, 25- and 100-fold molar excess of unlabeled DNA.

B. Mutational analysis of MEE2. The EMSA was carried out using radiolabeled MEE2 and parasite nuclear extract. Competition was carried out in presence of a 25- and 100-fold molar excess of unlabeled DNA. The nucleotide sequences of wild-type and mutated MEE2 elements are indicated on the right. The ATAGATTA core motif is underlined. Mutated 8mers are highlighted in red (see Fig. S3A for competition with MEE2-mut6). The MEE2-related element upstream of PF07_0048 is shown at the bottom and differences compared to MEE2 are highlighted in red.

Next, we asked if the MEE2 element also occurs upstream of other *var* genes. We inspected all *var* upstream sequences (-600 to -1 relative to the start ATG) and identified a perfect or slightly deviated MEE2 core motif with the consensus sequence (A/T)(A/T)(A/T)GA(T/A)TA in 44 (73%) out of all 60 *var* genes. Strikingly, in all but four cases this motif (1) is conserved in terms of orientation and position relative to the ATG start codon; (2) is embedded in an overall highly similar sequence context including a characteristic poly-dT stretch; and (3) occurs in *upsB*-, *upsC*-, *upsB*/*C*- and *upsB*/*A*-type *var* genes (Fig. S4). The remaining four core motifs were found in one *upsB*/*C* and three *upsA*-type upstream sequences but they did not share these characteristics; they occurred in a different sequence context and relative position/orientation. In EMSA experiments, the MEE2-like motif derived from another *upsC var* gene (PF07_0048), in which six nucleotide positions are changed compared to MEE2 including one substitution in the core motif, competed as efficiently as the wild-type MEE2 motif (Fig. 5B). Similarly, the element found upstream of an *upsB*-type *var* gene (PFL0005w), in which 19 positions are altered including two in the core motif, competed albeit with lower efficiency (Fig. S3B). In contrast, competitors derived from a *var upsA* (PFD1235w) and a *var*-unrelated *rif* (PFB0035c) upstream region, in which a AT(A/T)GATTA core motif is present at the same relative position as in MEE2, failed to inhibit formation of the MEE2-protein complex (Fig. S3C).

Together, our results show that the MEE2-interacting factor (MIF) also binds to related motifs found in a large proportion of *var* upstream regions. Interestingly, however, MIF does not bind to unrelated sequences that contain a perfectly conserved 8bp MEE2 core motif. Hence, this core motif is necessary but not sufficient for binding and the local *var* upstream sequence context plays an important role in mediating stable and sequence-specific complex formation.

2.4. Discussion

The importance of mutually exclusive transcription of gene families is exemplified by antigenic variation in unicellular pathogens as a prime strategy to secure survival and transmission. In *Trypanosoma brucei*, the causing agent of African sleeping sickness, mutually exclusive transcription of variant surface glycoprotein genes is carried out by an extra-nucleolar RNA polI-containing body (Navarro and Gull, 2001). Another paradigm of mutual exclusion is that of singular odorant receptor (OR) gene choice in individual olfactory neurons in mammals (McClintock, 2010). Here, exclusive transcription of one out of over a

thousand OR genes involves regulatory DNA elements both upstream and in the coding regions (Fuss *et al.*, 2007;Lomvardas *et al.*, 2006;Nguyen *et al.*, 2007;Qasba and Reed, 1998;Vassalli *et al.*, 2002), and a negative protein feedback mechanism (Lewcock and Reed, 2004;Serizawa *et al.*, 2003;Shykind *et al.*, 2004). In addition, and in remarkable analogy to mutually exclusive *var* regulation, Lomvardas and colleagues recently described a functional association of H3K9me3 and H3K4me3 with silenced and active OR loci, respectively (Magklara *et al.*, 2011). These important discoveries notwithstanding, we still lack detailed knowledge as to how mutually exclusive transcription is achieved in any system. In this study, we developed and successfully applied a complementary functional approach to study mutual exclusion in *P. falciparum var* gene transcription. For the first time, we identified *cis*-acting entities as important mediators of *var* gene activation and singular gene choice.

var gene transcription is mediated by RNA polII and occurs stage-specifically by activation in ring stage parasites and subsequent repression or poising during the rest of the IDC (Kyes *et al.*, 2007;Lopez-Rubio *et al.*, 2007). Here, we identified an UAS element essential for *upsC* promoter activation. The position of this element upstream of the natural TSS, and the competence to activate transcription from a heterologous promoter, are attributes inherently associated with the role of UAS elements in transcriptional activation (Levine and Tjian, 2003). Our results are therefore consistent with the sequence-specific recruitment of a transcriptional activator by the UAS to orchestrate the assembly of the pre-initiation complex (PIC) and/or to activate RNA polII-dependent transcription. Interestingly, the fact that this element functions autonomously in a euchromatic context implies a ubiquitous rather than spatially restricted distribution of the transcriptional activator involved, which somewhat precludes a restricted role for this factor in mutually exclusive *var* activation.

The current model of mutually exclusive *var* transcription postulates the existence of a physically restricted perinuclear zone dedicated to the expression of a single *var* gene (Duraisingh *et al.*, 2005;Dzikowski *et al.*, 2007;Lopez-Rubio *et al.*, 2009;Ralph *et al.*, 2005;Voss *et al.*, 2006;Voss *et al.*, 2007). Activation requires entry into this zone with concomitant substitution of the formerly active locus, linked to the removal of H3K9me3/PfHP1 and deposition of H3K9ac and H3K4me2/3 marks predominantly along the region downstream of the TSS (Lopez-Rubio *et al.*, 2007;Perez-Toledo *et al.*, 2009). We identified a deletion downstream of the TSS as the common denominator of all four promoter variants that escaped mutually exclusive activation. Unlike full-length promoters, activation of promoters lacking this region did not occur at the expense of, but in parallel to, the

transcription of an endogenous *var* gene. Notably, this deletion did not alter the relative activity of the promoter showing that the processes of promoter activation and mutually exclusive recognition are uncoupled from each other. The specific binding of a nuclear factor or complex (MIF) to a *cis*-acting sequence motif present in this region (MEE2) corroborates this hypothesis and suggests an important role for this DNA-protein interaction in mutually exclusive promoter activation. The presence of MEE2-related motifs in a large subset of *var* genes provides circumstantial evidence for a conserved mechanism of singular *var* gene choice. Although the exact function of this interaction remains to be discovered, binding of MIF to the mutual exclusion element may earmark *var* loci for mutually exclusive activation. Additional experiments tailored towards identifying MIF and dissecting the exact function of this interaction in *var* regulation are now required to test this hypothesis. In this context it is worth mentioning that the 47bp MEE2 sequence does not contain any obvious ApiAP2 transcription factor binding motifs (Campbell *et al.*, 2010).

Using promoter deletion analyses combined with ectopic insertion of *var* elements into a euchromatic locus we were able to systematically reconstruct some of the control steps of *var* gene activation and mutual exclusion. Based on these novel findings, and by integrating current knowledge, we propose a speculative mechanistic model for mutually exclusive *var* gene activation (Fig. 6). The position of *var* loci in heterochromatic perinuclear clusters prevents accessibility to specific and general transcription factors and this is probably the most important determinant of transcriptional inactivity (Duraisingh *et al.*, 2005;Flueck *et al.*, 2009;Freitas-Junior *et al.*, 2000;Freitas-Junior *et al.*, 2005;Lopez-Rubio *et al.*, 2009;Perez-Toledo *et al.*, 2009;Ralph *et al.*, 2005;Voss *et al.*, 2006). The MEE2-interacting factor or complex MIF may bind downstream of the TSS to reinforce repression and/or to prevent or reduce leaky transcription from silenced loci. Such a function may be crucial in keeping *var* genes repressed that are positioned within euchromatic zones at the nuclear periphery (Ralph *et al.*, 2005). Singular *var* gene choice may occur through the recognition of the MEE2/MIF complex, or an alternative *var* locus-specific sequence tag, by the unique *var* gene expression site (VES) (Duraisingh *et al.*, 2005;Dzikowski *et al.*, 2007;Lopez-Rubio *et al.*, 2009;Voss *et al.*, 2006). Once locked in, the VES may trigger the exchange of H3K9me3/PfHP1 with H3K4me2/3 and H3K9ac marks and the dissociation of the repressive MIF complex. Physical association of the active *var* locus with the VES may also play a crucial role in epigenetic memory, i.e. in keeping the *var* gene in place for re-activation in daughter cells (Lopez-Rubio *et al.*, 2007). In this context, it is tempting to speculate that the recently identified histone methyltransferase PfSET10 (Volz *et al.*, 2012) may be one component of the VES compartment.

This model proposes a novel logic in mutually exclusive gene expression and provides us with an informed working hypothesis for further functional dissection of the mechanisms orchestrating singular *var* gene choice. In particular, targeted identification of the proteins or protein complexes interacting with the regulatory elements characterised in this study will be a promising and exciting avenue to pursue. Detailed insight into this complex regulatory system is important for our understanding of immune evasion and virulence of *P. falciparum* and other pathogens. Furthermore, our results will also help to understand conceptually similar processes in other organisms.

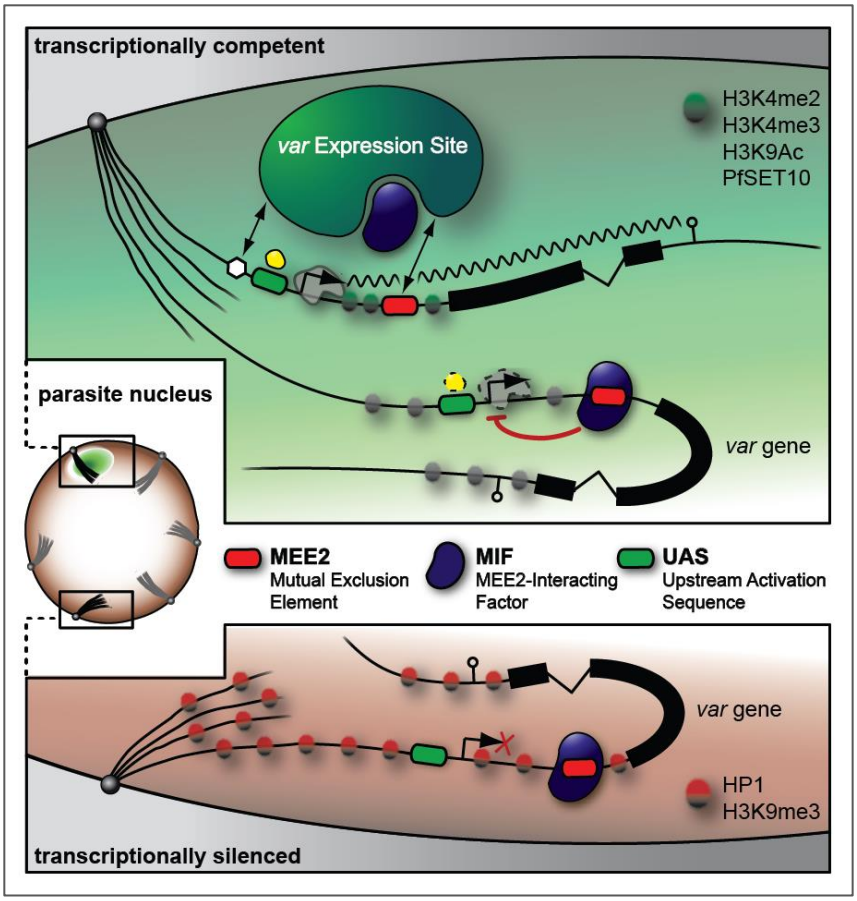


Fig. 6. A novel model for singular *var* gene choice.
A chromosome end cluster located in a transcriptionally permissive perinuclear region is schematically depicted on top. The unique *var* gene expression site (VES) recognises a single *var* gene through specific interaction with unknown DNA motifs (white hexagon) and/or the MEE2 element itself (red oval). This interaction leads to dissociation of the MIF complex (blue) concomitant with the establishment of a permissive chromatin conformation (green circles) to facilitate RNA polII-dependent transcriptional initiation and/or elongation. This process involves deposition and maintenance of permissive histone modifications through modifying enzymes such as PfSET10 (Volz *et al*., 2012) as well as interactions between unknown transcription factors (yellow) and the UAS (green oval). Additional *var* genes within this subnuclear domain are excluded from the VES and protected from illegitimate transcription. Here, the function of MIF may be to block transcriptional elongation or to prevent transcriptional initiation or PIC assembly on the core promoter. *var* genes in heterochromatic perinuclear zones that are silenced primarily through their association with H3K9me3/PfHP1 are shown below.

2.5. Experimental Procedures

Parasite culture and transfection

P. falciparum 3D7 parasites were cultured as described previously (Trager and Jenson, 1978). Growth synchronisation was achieved by repeated sorbitol lysis (Lambros and Vanderberg, 1979). Transfections were performed as described (Voss *et al.*, 2006). Parasites were selected on 2.5µg/ml blasticidin-S-HCl and 4nM WR99210. Transfection constructs are described in Supporting Experimental Procedures.

Quantitative reverse transcription PCR

qPCR was performed on reverse transcribed total RNA and gDNA isolated from synchronous parasite cultures. A detailed protocol, relative transcript calculation and primer sequences are provided in Supporting Experimental Procedures and Table S1.

Southern and Northern blot analysis

gDNA was digested with appropriate restriction enzymes overnight and separated in 0.5xTBE-buffered 0.7% agarose gels. Total RNA was isolated from saponin-released parasites using TriReagent (Ambion). RNA was glyoxylated for 1h at 55°C in five volumes glyoxal reaction mixture and electrophoresis was performed using 1xBPTE-buffered 1.5% agarose gels (Sambrook and Russell, 2001). Blots were probed with ^{32}P-dATP-labeled h*dhfr*, *kahrp* and *hsp86* PCR fragments. Membranes were stripped by boiling in 0.1% SDS for 15min in between hybridisations.

Western blot analysis

Detection of hDHFR-GFP and GAPDH (loading control) was performed on whole cell lysates. Primary antibody dilutions were: mouse anti-GFP (Roche Diagnostics, 11814460001), 1:500; mouse anti-GAPDH 1-10B (kind gift of Claudia Daubenberger), 1:20’000. PfEMP1 was extracted from trophozoite-infected RBC pellets (Triton X-100-insoluble/SDS soluble fraction) as described (van Schravendijk *et al.*, 1993). Extracts were separated by SDS-PAGE using 5% polyacrylamide gels using Tris-glycine or Tris-acetate buffers. PfEMP1 was detected using the monoclonal mouse anti-PfEMP1 antibody 1B/6H-1 (Duffy *et al.*, 2002), 1:500.

Electromobility shift assay

High salt nuclear extracts and EMSAs were prepared and carried out as described (Voss *et al.*, 2002) with the following modifications. Proteins were extracted with 500mM KCl and incubated with 20fmol of radiolabeled probe in 1xEMSA buffer in presence of 200ng of poly(dA-dT) as nonspecific competitor. Complementary oligonucleotide sequences used to generate double stranded probes and competitors are listed in Table S1.

2.6. Acknowledgements

We are grateful to Dania Mueller for help in qRT-PCR experiments and Sandra Birrer for help in cloning and transfection. We thank Tim-Wolf Gilberger for critical reading of the manuscript. N.M.B.B. received a Boehringer Ingelheim PhD fellowship (www.bifonds.de). This work is supported by the Swiss National Science Foundation (PP00A-110835; PP00P3_130203; www.snf.ch). The authors declare that no conflict of interest exists.

2.7. References

Baruch, D. I., Gormely, J. A., Ma, C., Howard, R. J., and Pasloske, B. L. (1996) Plasmodium falciparum erythrocyte membrane protein 1 is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. *Proc Natl Acad Sci U S A* **93:** 3497-3502.

Beeson, J. G. and Duffy, P. E. (2005) The immunology and pathogenesis of malaria during pregnancy. *Curr Top Microbiol Immunol* **297:** 187-227.

Biggs, B. A., Gooze, L., Wycherley, K., Wollish, W., Southwell, B., Leech, J. H. *et al.* (1991) Antigenic variation in Plasmodium falciparum. *Proc Natl Acad Sci U S A* **88:** 9171-9174.

Calderwood, M. S., Gannoun-Zaki, L., Wellems, T. E., and Deitsch, K. W. (2003) Plasmodium falciparum var genes are regulated by two regions with separate promoters, one upstream of the coding region and a second within the intron. *J Biol Chem* **278:** 34125-34132.

Campbell, T. L., De Silva, E. K., Olszewski, K. L., Elemento, O., and Llinas, M. (2010) Identification and genome-wide prediction of DNA binding specificities for the ApiAP2 family of regulators from the malaria parasite. *PLoS Pathog* **6:** e1001165.

Chookajorn, T., Dzikowski, R., Frank, M., Li, F., Jiwani, A. Z., Hartl, D. L. *et al.* (2007) Epigenetic memory at malaria virulence genes. *Proc Natl Acad Sci U S A* **104:** 899-902.

Deitsch, K. W., Calderwood, M. S., and Wellems, T. E. (2001) Malaria. Cooperative silencing elements in var genes. *Nature* **412:** 875-876.

Deitsch, K. W., del, P. A., and Wellems, T. E. (1999) Intra-cluster recombination and var transcription switches in the antigenic variation of Plasmodium falciparum. *Mol Biochem Parasitol* **101:** 107-116.

Deitsch, K. W., Lukehart, S. A., and Stringer, J. R. (2009) Common strategies for antigenic variation by bacterial, fungal and protozoan pathogens. *Nat Rev Microbiol* **7:** 493-503.

Duffy, M. F., Brown, G. V., Basuki, W., Krejany, E. O., Noviyanti, R., Cowman, A. F. *et al.* (2002) Transcription of multiple var genes by individual, trophozoite-stage Plasmodium falciparum cells expressing a chondroitin sulphate A binding phenotype. *Mol Microbiol* **43:** 1285-1293.

Duraisingh, M. T., Voss, T. S., Marty, A. J., Duffy, M. F., Good, R. T., Thompson, J. K. *et al.* (2005) Heterochromatin silencing and locus repositioning linked to regulation of virulence genes in Plasmodium falciparum. *Cell* **121:** 13-24.

Dzikowski, R. and Deitsch, K. W. (2009) Genetics of antigenic variation in Plasmodium falciparum. *Curr Genet* **55:** 103-110.

Dzikowski, R., Frank, M., and Deitsch, K. (2006) Mutually exclusive expression of virulence genes by malaria parasites is regulated independently of antigen production. *PLoS Pathog* **2:** e22.

Dzikowski, R., Li, F., Amulic, B., Eisberg, A., Frank, M., Patel, S. *et al.* (2007) Mechanisms underlying mutually exclusive expression of virulence genes by malaria parasites. *EMBO Rep* **8:** 959-965.

Flueck, C., Bartfai, R., Volz, J., Niederwieser, I., Salcedo-Amaya, A. M., Alako, B. T. *et al.* (2009) Plasmodium falciparum heterochromatin protein 1 marks genomic loci linked to phenotypic variation of exported virulence factors. *PLoS Pathog* **5:** e1000569.

Frank, M., Dzikowski, R., Costantini, D., Amulic, B., Berdougo, E., and Deitsch, K. (2006) Strict pairing of var promoters and introns is required for var gene silencing in the malaria parasite Plasmodium falciparum. *J Biol Chem* **281:** 9942-9952.

Freitas-Junior, L. H., Bottius, E., Pirrit, L. A., Deitsch, K. W., Scheidig, C., Guinet, F. *et al.* (2000) Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of P. falciparum. *Nature* **407:** 1018-1022.

Freitas-Junior, L. H., Hernandez-Rivas, R., Ralph, S. A., Montiel-Condado, D., Ruvalcaba-Salazar, O. K., Rojas-Meza, A. P. *et al.* (2005) Telomeric heterochromatin propagation and histone acetylation control mutually exclusive expression of antigenic variation genes in malaria parasites. *Cell* **121:** 25-36.

Fuss, S. H., Omura, M., and Mombaerts, P. (2007) Local and cis effects of the H element on expression of odorant receptor genes in mouse. *Cell* **130:** 373-384.

Gannoun-Zaki, L., Jost, A., Mu, J., Deitsch, K. W., and Wellems, T. E. (2005) A silenced Plasmodium falciparum var promoter can be activated in vivo through spontaneous deletion of a silencing element in the intron. *Eukaryot Cell* **4:** 490-492.

Gardner, J. P., Pinches, R. A., Roberts, D. J., and Newbold, C. I. (1996) Variant antigens and endothelial receptor adhesion in Plasmodium falciparum. *Proc Natl Acad Sci U S A* **93:** 3503-3508.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W. *et al.* (2002) Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature* **419:** 498-511.

Howitt, C. A., Wilinski, D., Llinas, M., Templeton, T. J., Dzikowski, R., and Deitsch, K. W. (2009) Clonally variant gene families in Plasmodium falciparum share a common activation factor. *Mol Microbiol* **73:** 1171-1185.

Kyes, S., Christodoulou, Z., Pinches, R., Kriek, N., Horrocks, P., and Newbold, C. (2007) Plasmodium falciparum var gene expression is developmentally controlled at the level of RNA polymerase II-mediated transcription initiation. *Mol Microbiol* **63:** 1237-1247.

Lambros, C. and Vanderberg, J. P. (1979) Synchronization of Plasmodium falciparum erythrocytic stages in culture. *J Parasitol* **65:** 418-420.

Lanzer, M., de, B. D., and Ravetch, J. V. (1992) A sequence element associated with the Plasmodium falciparum KAHRP gene is the site of developmentally regulated protein-DNA interactions. *Nucleic Acids Res* **20:** 3051-3056.

Lavstsen, T., Salanti, A., Jensen, A. T., Arnot, D. E., and Theander, T. G. (2003) Sub-grouping of Plasmodium falciparum 3D7 var genes based on sequence analysis of coding and non-coding regions. *Malar J* **2:** 27.

Leech, J. H., Barnwell, J. W., Miller, L. H., and Howard, R. J. (1984) Identification of a strain-specific malarial antigen exposed on the surface of Plasmodium falciparum-infected erythrocytes. *J Exp Med* **159:** 1567-1575.

Levine, M. and Tjian, R. (2003) Transcription regulation and animal diversity. *Nature* **424:** 147-151.

Lewcock, J. W. and Reed, R. R. (2004) A feedback mechanism regulates monoallelic odorant receptor expression. *Proc Natl Acad Sci U S A* **101:** 1069-1074.

Lomvardas, S., Barnea, G., Pisapia, D. J., Mendelsohn, M., Kirkland, J., and Axel, R. (2006) Interchromosomal interactions and olfactory receptor choice. *Cell* **126:** 403-413.

Lopez-Rubio, J. J., Gontijo, A. M., Nunes, M. C., Issar, N., Hernandez, R. R., and Scherf, A. (2007) 5' flanking region of var genes nucleate histone modification patterns linked to phenotypic inheritance of virulence traits in malaria parasites. *Mol Microbiol* **66:** 1296-1305.

Lopez-Rubio, J. J., Mancio-Silva, L., and Scherf, A. (2009) Genome-wide analysis of heterochromatin associates clonally variant gene regulation with perinuclear repressive centers in malaria parasites. *Cell Host Microbe* **5:** 179-190.

MacPherson, G. G., Warrell, M. J., White, N. J., Looareesuwan, S., and Warrell, D. A. (1985) Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *Am J Pathol* **119:** 385-401.

Magklara, A., Yen, A., Colquitt, B. M., Clowney, E. J., Allen, W., Markenscoff-Papadimitriou, E. *et al.* (2011) An epigenetic signature for monoallelic olfactory receptor expression. *Cell* **145:** 555-570.

Marty, A. J., Thompson, J. K., Duffy, M. F., Voss, T. S., Cowman, A. F., and Crabb, B. S. (2006) Evidence that Plasmodium falciparum chromosome end clusters are cross-linked by protein and are the sites of both virulence gene silencing and activation. *Mol Microbiol* **62:** 72-83.

McClintock, T. S. (2010) Achieving singularity in mammalian odorant receptor gene choice. *Chem Senses* **35:** 447-457.

Morrison, L. J., Marcello, L., and McCulloch, R. (2009) Antigenic variation in the African trypanosome: molecular mechanisms and phenotypic complexity. *Cell Microbiol* **11:** 1724-1734.

Navarro, M. and Gull, K. (2001) A pol I transcriptional body associated with VSG mono-allelic expression in Trypanosoma brucei. *Nature* **414:** 759-763.

Nguyen, M. Q., Zhou, Z., Marks, C. A., Ryba, N. J., and Belluscio, L. (2007) Prominent roles for odorant receptor coding sequences in allelic exclusion. *Cell* **131:** 1009-1017.

O'Donnell, R. A., Freitas-Junior, L. H., Preiser, P. R., Williamson, D. H., Duraisingh, M., McElwain, T. F. *et al.* (2002) A genetic screen for improved plasmid segregation reveals a role for Rep20 in the interaction of Plasmodium falciparum chromosomes. *EMBO J* **21:** 1231-1239.

Perez-Toledo, K., Rojas-Meza, A. P., Mancio-Silva, L., Hernandez-Cuevas, N. A., Delgadillo, D. M., Vargas, M. *et al.* (2009) Plasmodium falciparum heterochromatin protein 1 binds to tri-methylated histone 3 lysine 9 and is linked to mutually exclusive expression of var genes. *Nucleic Acids Res* **37:** 2596-2606.

Pongponratn, E., Riganti, M., Punpoowong, B., and Aikawa, M. (1991) Microvascular sequestration of parasitized erythrocytes in human falciparum malaria: a pathological study. *Am J Trop Med Hyg* **44:** 168-175.

Prucca, C. G. and Lujan, H. D. (2009) Antigenic variation in Giardia lamblia. *Cell Microbiol* **11:** 1706-1715.

Qasba, P. and Reed, R. R. (1998) Tissue and zonal-specific expression of an olfactory receptor transgene. *J Neurosci* **18:** 227-236.

Ralph, S. A., Scheidig-Benatar, C., and Scherf, A. (2005) Antigenic variation in Plasmodium falciparum is associated with movement of var loci between subnuclear locations. *Proc Natl Acad Sci U S A* **102:** 5414-5419.

Reeder, J. C., Cowman, A. F., Davern, K. M., Beeson, J. G., Thompson, J. K., Rogerson, S. J. *et al.* (1999) The adhesion of Plasmodium falciparum-infected erythrocytes to chondroitin sulfate A is mediated by P. falciparum erythrocyte membrane protein 1. *Proc Natl Acad Sci U S A* **96:** 5198-5202.

Roberts, D. J., Craig, A. G., Berendt, A. R., Pinches, R., Nash, G., Marsh, K. *et al.* (1992) Rapid switching to multiple antigenic and adhesive phenotypes in malaria. *Nature* **357:** 689-692.

Salcedo-Amaya, A. M., van Driel, M. A., Alako, B. T., Trelle, M. B., van den Elzen, A. M., Cohen, A. M. *et al.* (2009) Dynamic histone H3 epigenome marking during the intraerythrocytic cycle of Plasmodium falciparum. *Proc Natl Acad Sci U S A* **106:** 9655-9660.

Sambrook, J. and Russell, D. (2001) *Molecular Cloning: A Laboratory Manual*. CSHL Press.

Scherf, A., Hernandez-Rivas, R., Buffet, P., Bottius, E., Benatar, C., Pouvelle, B. *et al.* (1998) Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum. *EMBO J* **17:** 5418-5426.

Serizawa, S., Miyamichi, K., Nakatani, H., Suzuki, M., Saito, M., Yoshihara, Y. *et al.* (2003) Negative feedback regulation ensures the one receptor-one olfactory neuron rule in mouse. *Science* **302:** 2088-2094.

Shykind, B. M., Rohani, S. C., O'Donnell, S., Nemes, A., Mendelsohn, M., Sun, Y. *et al.* (2004) Gene switching and the stability of odorant receptor gene choice. *Cell* **117:** 801-815.

Smith, J. D., Chitnis, C. E., Craig, A. G., Roberts, D. J., Hudson-Taylor, D. E., Peterson, D. S. *et al.* (1995) Switches in expression of Plasmodium falciparum var genes correlate

with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell* **82:** 101-110.

Su, X. Z., Heatwole, V. M., Wertheimer, S. P., Guinet, F., Herrfeldt, J. A., Peterson, D. S. *et al.* (1995) The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. *Cell* **82:** 89-100.

Trager, W. and Jenson, J. B. (1978) Cultivation of malarial parasites. *Nature* **273:** 621-622.

van Schravendijk, M. R., Pasloske, B. L., Baruch, D. I., Handunnetti, S. M., and Howard, R. J. (1993) Immunochemical characterization and differentiation of two approximately 300-kD erythrocyte membrane-associated proteins of Plasmodium falciparum, PfEMP1 and PfEMP3. *Am J Trop Med Hyg* **49:** 552-565.

Vassalli, A., Rothman, A., Feinstein, P., Zapotocky, M., and Mombaerts, P. (2002) Minigenes impart odorant receptor-specific axon guidance in the olfactory bulb. *Neuron* **35:** 681-696.

Volz, J. C., Bartfai, R., Petter, M., Langer, C., Josling, G. A., Tsuboi, T. *et al.* (2012) PfSET10, a Plasmodium falciparum methyltransferase, maintains the active var gene in a poised state during parasite division. *Cell Host Microbe* **11:** 7-18.

Voss, T. S., Healer, J., Marty, A. J., Duffy, M. F., Thompson, J. K., Beeson, J. G. *et al.* (2006) A var gene promoter controls allelic exclusion of virulence genes in Plasmodium falciparum malaria. *Nature* **439:** 1004-1008.

Voss, T. S., Mini, T., Jenoe, P., and Beck, H. P. (2002) Plasmodium falciparum possesses a cell cycle-regulated short type replication protein A large subunit encoded by an unusual transcript. *J Biol Chem* **277:** 17493-17501.

Voss, T. S., Thompson, J. K., Waterkeyn, J., Felger, I., Weiss, N., Cowman, A. F. *et al.* (2000) Genomic distribution and functional characterisation of two distinct and conserved Plasmodium falciparum var gene 5' flanking sequences. *Mol Biochem Parasitol* **107:** 103-115.

Voss, T. S., Tonkin, C. J., Marty, A. J., Thompson, J. K., Healer, J., Crabb, B. S. *et al.* (2007) Alterations in local chromatin environment are involved in silencing and activation of subtelomeric var genes in Plasmodium falciparum. *Mol Microbiol* **66:** 139-150.

Witmer, K., Schmid, C. D., Brancucci, N. M., Luah, Y. H., Preiser, P. R., Bozdech, Z. *et al.* (2012) Analysis of subtelomeric virulence gene families in Plasmodium falciparum by comparative transcriptional profiling. *Mol Microbiol* **84**: 243-59.

World Health Organisation (2010) *World Malaria Report 2010*. Geneva: WHO Press.

Zhang, Q., Huang, Y., Zhang, Y., Fang, X., Claes, A., Duchateau, M. *et al.* (2011) A critical role of perinuclear filamentous actin in spatial repositioning and mutually exclusive expression of virulence genes in malaria parasites. *Cell Host Microbe* **10:** 451-463.

2.8. Supporting Information

2.8.1. Supporting Experimental Procedures

Transfection constructs

All transfection constructs generated in this study are derivatives of pBK_{min} that was itself obtained by replacing the *cam* promoter in pBcam (Witmer *et al*., 2012) with the 1115bp minimal *kahrp* promoter (K_{min}) amplified from 3D7 gDNA and *Bgl*II/*Not*I restriction. Constructs pBC and pBC1 to pBC8 were generated by replacing K_{min} with *Bgl*II/*Not*I-digested *upsC* upstream sequences (PFL1960w) amplified from pCAT5B1 (Voss *et al*., 2000). upsC-*kahrp* hybrid promoters were obtained by cloning *BamH*I-digested upsC fragments into *Bgl*II-digested pBK_{min}. The *mahrp1* promoter was amplified from gDNA and cloned into *Bgl*II/*Not*I-digested pBK_{min} to obtain pBM. Importantly, the parental vector pBcam contains the 5bp AAAACA sequence, which naturally occurs directly upstream of the PFL1960w *var* ATG, upstream of the h*dhfr-gfp* ATG. This ensures an identical sequence context for translational initiation for all constructs. All primer sequences are listed in Table S1.

Quantitative reverse transcription PCR

Pre-synchronised parasite cultures were synchronised twice 16 hours apart to obtain an eight hour growth window. Total RNA was isolated using Tri Reagent (Ambion) and further purified using the RNeasy Plus Mini Kit (Qiagen) for removal of gDNA. Residual gDNA was digested with TURBO DNA-*free*™ (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 of h*dhfr-gfp*, *kahrp*, PF13_0170 (glutaminyl-tRNA synthetase) and *msp8* 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 12ul. Plasmid copy numbers were determined by qPCR on gDNA isolated from the same parasite samples and calculated by dividing the absolute h*dhfr-gfp* copy numbers by the average value obtained for *msp8* and PF13_0170. All reactions were run in duplicate or triplicate yielding virtually identical Ct values. Serial dilutions of gDNA and plasmid DNA were used as standards for absolute quantification. Relative "transcripts per parasite" were calculated by

normalisation against the house-keeping gene PF13_0170 or *msp8*. Relative "transcripts per promoter" were calculated by dividing the relative "transcripts per parasite" by the average number of plasmid copies. Primer sequences are listed in Table S1.

2.8.2. Supporting Figure and Table Legends

Fig. S1. Southern analysis of gDNA isolated from parasites presented in Fig. 2.

A. Autoradiographs of Southern blots showing episomal maintenance or plasmid integration into the endogenous *kahrp* locus in 3D7/pBK$_{min}$ and 3D7/pBK$_{min}$-RI. gDNA was digested with *Bgl*II and *Hind*III. Blots were probed with a radiolabeled *kahrp* fragment. E, episomal; I, integrated.

B. Autoradiographs of Southern blots showing episomal maintenance or plasmid integration into the endogenous *kahrp* locus in 3D7/pBC1K$_{min}$, 3D7/pBC2K$_{min}$ and 3D7/pBC3K$_{min}$. gDNA was digested with *Bgl*II and *Hind*III. Blots were probed with a radiolabeled *kahrp* fragment.

C. Schematic map of the endogenous *kahrp* locus.

D-F. Schematic maps of the integration events in 3D7/pBK$_{min}$ (D), 3D7/pBC1K$_{min}$ and 3D7/pBC2K$_{min}$ (E), and 3D7/pBC3K$_{min}$ (F). *Bgl*II and *Hind*III restriction sites and length of the corresponding fragments are indicated.

Fig. S2. Transcriptional initiation form an alternative *upsC* upstream TSS.

The promoters in pBC and pBC4 are schematically depicted on top. Semi-quantitative analysis of protein and transcript abundance by Western and Northern blot in a time-course experiment. Total protein and RNA were harvested simultaneously from synchronised 3D7/pBC and 3D7/pBC4 parasites at three consecutive time points during intra-erythrocytic development (ring stages, 8-18hpi; late ring stages/early trophozoites, 16-26hpi; late trophozoites/early schizonts, 24-34hpi). Expression of hDHFR-GFP and GAPDH (loading control) was detected with anti-GFP and anti-GAPDH antibodies, respectively (upper panels). Steady-state h*dhfr-gfp* and *hsp86* (loading control) transcripts were detected using radiolabeled h*dhfr* and *hsp86* probes, respectively.

Fig. S3. Competition EMSAs. All EMSAs were carried out using radiolabeled MEE2 and parasite nuclear extract.

A. Mutational analysis of MEE2. Competition was carried out in presence of a 25- and 100-fold molar excess of unlabeled DNA. The nucleotide sequences of wild-type and mutated MEE2 elements are indicated on the right. The ATAGATTA core motif is underlined. Mutated 8mers are highlighted in red.

B. Competition of the MEE2 complex by a MEE2-related upsB sequence element. Competition was carried out in presence of a 25-, 100-, 250- and 500-fold molar excess of unlabeled DNA. The nucleotide sequences of wild-type and scrambled MEE2 and the MEE2-related upsB element are indicated on the right. The ATAGATTA core motif is underlined. The differences in the upsB-derived motif compared to MEE2 are highlighted in red.

C. The ATAGATTA core motif is not sufficient for complex formation. Competition was carried out in presence of a 25-, 100- and 500-fold molar excess of unlabeled DNA. The ATAGATTA core motif is underlined. The nucleotide sequences of wild-type and scrambled MEE2 and two unrelated sequence elements that contain the ATAGATTA core motif are indicated on the right.

Fig. S4. The MEE2 core motif occurs in a conserved position upstream of 44 *var* genes.

A. The schematic shows the presence and relative position of the (A/T)(A/T)(A/T)GA(A/T)TA consensus sequence found upstream of 44 *var* genes. This motif forms the core of the 47bp MEE2 element that is bound by a nuclear factor in a sequence-specific manner (see Figs. 4 and S3). Red boxes indicate the position of the motif in each upstream region. Numbers on the right represent the position of the first nucleotide of the motif relative to the translation initiation ATG. Gene accession numbers were retrieved from PlasmoDB version 7.2 (www.plasmoDB.org) and are indicated on the left. The colour code clusters *var* genes into the different *var* gene subgroups upsA, upsB, upsC, upsE, upsB/C, and upsB/A (Lavstsen et al., 2003).

B. Alignment of MEE2-related sequences that are centred around the (A/T)(A/T)(A/T)GA(A/T)TA core consensus element in 44 *var* upstream regions. The original MEE2 motif identified upstream of the upsC *var* gene PFL1960w is shown as the first sequence in the alignment. The local context of the MEE2-related core motifs shows a high level of sequence similarity that includes a prominent upstream poly-dT stretch. Gene accession numbers are indicated on the left and are colour-coded as in Fig. S4A. Orientation of the motif is indicated on the right (+, upper strand; -, lower strand). The red bar on top highlights the position of the core motif.

Table S1. All primers used in this study are listed. Restriction sites are indicated in bold.

	primer sequence (5`→3`)	RE sites
pBKmin		
kahrp-Rev-N-N	gatc**gcggccgc**ata**gctagc**gattctctaataattatgtacg	NotI, NheI
kahrp-Fwd-P-B-C	gatc**ctgcag**atgact**agatct**acgtgta**atcgat**cctaaaactgcatgtagtg	PstI, BglII, ClaI
upsC-Kmin hybrid promoters		
upsC-1_forw	cagt**ggatcc**atttcatcattattaaagtagag	BamHI
upsC-1_rev	cagt**ggatcc**ttctatctatattatctaccac	BamHI
upsC-2_forw	cagt**ggatcc**ttttttttctttgatgttgtac	BamHI
upsC-2_rev	cagt**ggatcc**atatttcatactaatattattcac	BamHI
upsC-3_forw	cagt**ggatcc**ccactacatggtattaccac	BamHI
upsC-3_rev	cagt**ggatcc**gttcgtgactacatgatgtc	BamHI
upsC deletion constructs		
upsC-F	cagt**agatct**ctttatgttggtacattatacatg	BglII
upsC-R	cagt**gcggccgc**ttttgtttttttgtttatcgttcg	NotI
upsC1-F	cagt**agatct**atagaaatattactgtttggag	BglII
upsC2-F	cagt**agatct**atttcatcattattaaagtagag	BglII
upsC3-F	cagt**agatct**atttattttcatagaaatgtgg	BglII
upsC4-R	cagt**gcggccgc**aaaaagaattataatcgaagaac	NotI
upsC5-R	cagt**gcggccgc**tgtttcttagtcgtactatatgtg	NotI
upsC6-R	cagt**gcggccgc**tatttaatactttatattatgtgg	NotI
upsC7-R	cagt**gcggccgc**atttatattaccatgatgccg	NotI
upsC8-R	cagt**gcggccgc**cacattattactctaatatgcg	NotI
qPCR		
PF13_0170F	tggctaggatatgattggaaagaaca	
PF13_0170R	tacggttctatttctatatggtgaatca	
gfpF	acacttgtcactactttcgcgtatggtcttc	
gfpR	accttcaaacttgacttcagcacgtgtcttgtagt	
kahrpF	acggatccggtgactccttcgat	
kahrpR	tggtgaacctgtggtgcttggtgat	
msp8F	tgacgcaaaagcaagggacaacaataataatgatga	
msp8R	tcatcgtcatcatcattatcatcatcatcacc	
hybridisation probes		
kahrpF	acggatccggtgactccttcgat	
kahrpR	tggtgaacctgtggtgcttggtgat	
hdhfrF	agctggatccgcggccgcaaaacatgcatggttcgctaaactg	
hdhfrR	agctgtcgacagcagcatcattcttctcatatacttcaa	
hsp86F	gaattgattagtaatgctagtg	
hsp86R	gtttcatccttagtaactgtg	
EMSA oligonucleotides		
MEE2-F	cagttgataatgatgtttttttttttgttatagattatgataacaagcttta	
MEE2-R	gacttaaagcttgttatcataatctataacaaaaaaaaaaacatcattatca	
MEE2-scrambled-F	agtatatagtattgaattgtaattgtcaatttctgtatttattgtta	
MEE2-scrambled-R	taacaataaatacagaaattgacaattacaattcaatactatatact	
MEE2-mut1-F	cggacgtcatgtttttttttgttatagattatgataacaatcttta	
MEE2-mut1-R	taaagattgttatcataatctataacaaaaaaaaaaacatgacgtccg	
MEE2-mut2-F	tgataatgcggacgtctttttgttatagattatgataacaagcttta	
MEE2-mut2-R	taaagcttgttatcataatctataacaaaaagacgtccgcattatca	
MEE2-mut3-F	tgataatgatgtttttcggacgtcatagattatgataacaagcttta	
MEE2-mut3-R	taaagcttgttatcataatctatgacgtccgaaaaacatcattatca	
MEE2-mut4-F	tgataatgatgtttttttttgttcggacgtctgataacaagcttta	
MEE2-mut4-R	taaagcttgttatcagacgtccgaacaaaaaaaaaacatcattatca	
MEE2-mut5-F	tgataatgatgtttttttttgttatagattacggacgtcagcttta	
MEE2-mut5-R	taaagctgacgtccgtaatctataacaaaaaaaaaacatcattatca	
MEE2-mut6-F	tgataatgatgtttttttttgttatagattatgataacacggacgt	
MEE2-mut6-R	acgtccgtgttatcataatctataacaaaaaaaaaacatcattatca	
MEE3-F	cagtacaagctttatgaatcgcatattagagtaataatgtgcatgcatgac	
MEE3-R	gactgtcatgcatgcacattattactctaatatgcgattcataaagcttgt	
PF07_0048-F	tgataatgatgttgtgttttgttaaagattatgataacaagatatat	
PF07_0048-R	atatatcttgttatcataatctttaacaaaacacaacatcattatca	
upsB-F	tgtaattgttgttttttttttgttagaatatttaaatttattataa	
upsB-R	ttataataaatttaaatattctaacaaaaaaaaaaacaacaattaca	
rifin-F	atattatggaactataataatattatagattaagaatatgtaattca	
rifin-R	tgaattacatattcttaatctataatattattatagttccataatat	
upsA-F	ttgaaatatatttagtgcatatatattgattataataattgtaatgt	
upsA-R	acattacaattattataatcaatatatatgcactaaatatatttcaa	
SPE1M-F	cacggacaaaaaaagtaaccgagaattattatatataaatat	
SPE1M-R	atatttatatataataattctcggttacttttttgtccgtg	

2.8.3. Supplemental References

Lavstsen, T., Salanti, A., Jensen, A. T., Arnot, D. E., and Theander, T. G. (2003) Sub-grouping of Plasmodium falciparum 3D7 var genes based on sequence analysis of coding and non-coding regions. *Malar J* **2:** 27.

Voss, T. S., Thompson, J. K., Waterkeyn, J., Felger, I., Weiss, N., Cowman, A. F. *et al.* (2000) Genomic distribution and functional characterisation of two distinct and conserved Plasmodium falciparum var gene 5' flanking sequences 109. *Mol Biochem Parasitol* **107:** 103-115.

Witmer, K., Schmid, C. D., Brancucci, N. M., Luah, Y. H., Preiser, P. R., Bozdech, Z. *et al.* (2012) Analysis of subtelomeric virulence gene families in Plasmodium falciparum by comparative transcriptional profiling. *Mol Microbiol* **84**: 243-59.

2.8.4. Supplementary Figures

Figure S1_Brancucci et al.

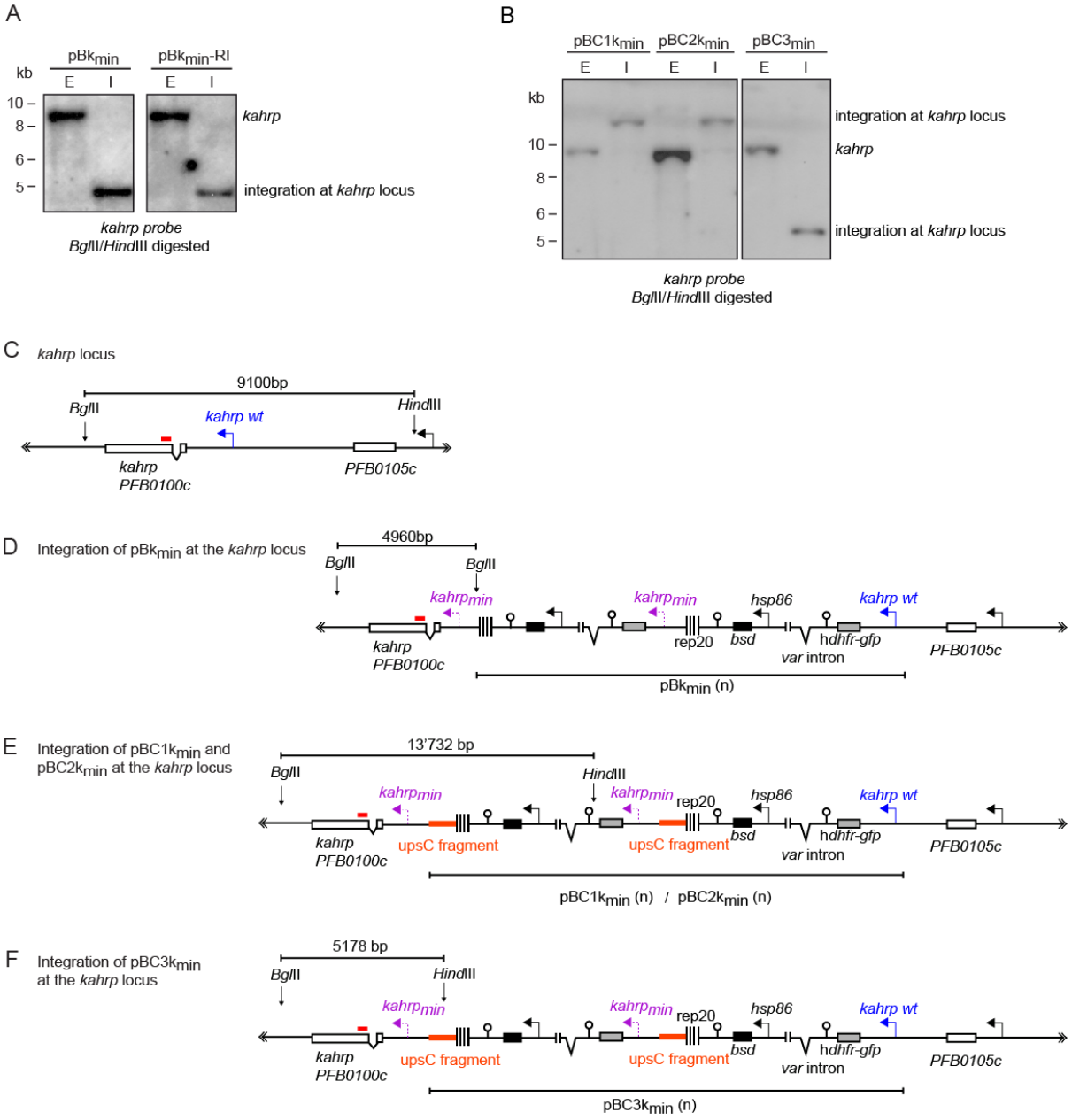


Figure S2_Brancucci et al.

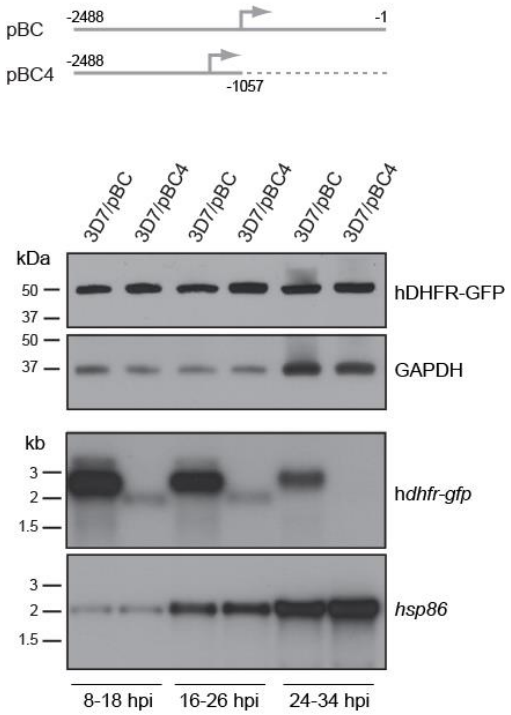
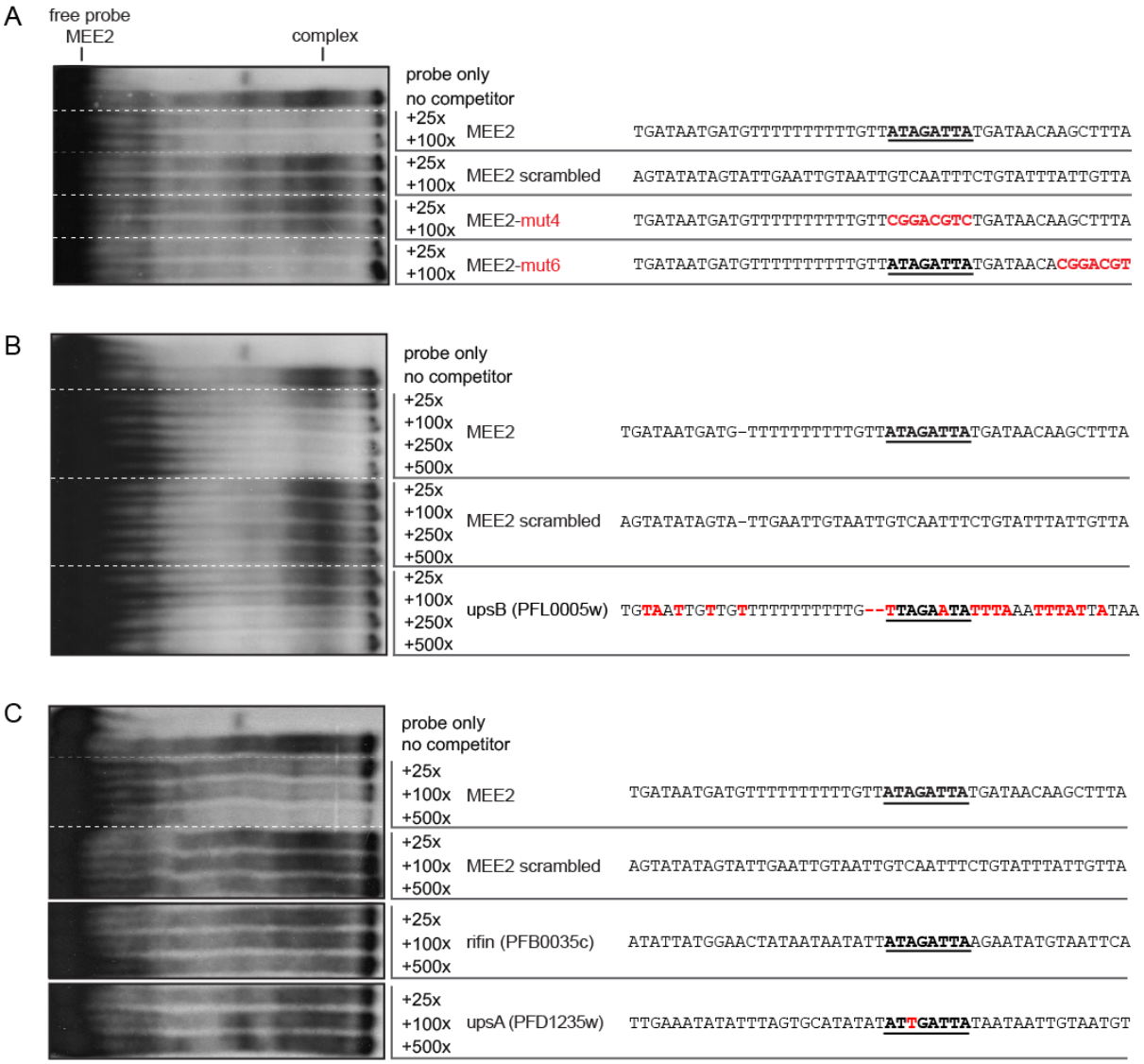


Figure S3_Brancucci et al.



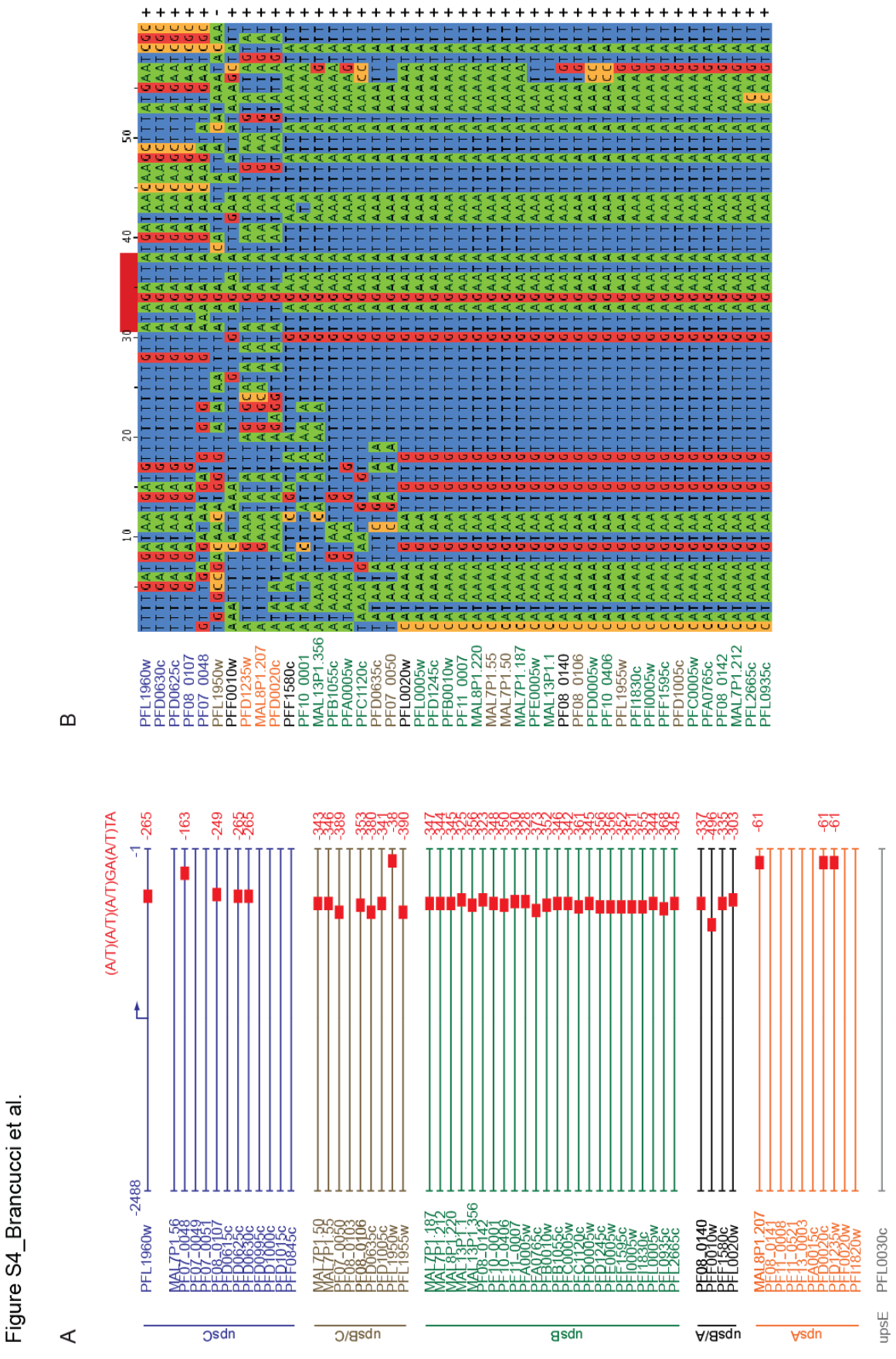


Figure S4_Brancucci et al.

Chapter 3

A *var* gene upstream element controls protein synthesis at the level of translation initiation in *Plasmodium falciparum*

Nicolas M. B. Brancucci[1,2], Christoph D. Schmid[1,2], and Till S. Voss[1,2,*]

[1]Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, 4051 Basel, Switzerland; [2]University of Basel, 4003 Basel, Switzerland

*Corresponding author: till.voss@unibas.ch; Phone: +41 61 284 81 61

3.1. Abstract

Clonally variant protein expression in the malaria parasite *Plasmodium falciparum* generates phenotypic variability and allows isogenic populations to adapt to environmental changes encountered during blood stage infection. The underlying regulatory mechanisms were best studied for the major virulence factor *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is encoded by the multicopy *var* gene family and only a single variant is expressed in individual parasites, a concept known as mutual exclusion or singular gene choice. *var* gene activation occurs *in situ* and is achieved through the escape of one locus from epigenetic silencing. Switches in *var* expression result in antigenic variation of PfEMP1 and immune evasion. Singular gene choice is controlled at the level of transcription initiation and *var* 5` upstream (*ups)* sequences harbour regulatory information that is essential for mutually exclusive transcription as well as for the trans-generational inheritance of the *var* activity profile. In addition, however, an mRNA element in the 5` untranslated region (5` UTR) was shown to reversibly repress translation of *var2csa* transcripts derived from the unique *upsE* promoter. Here, we extend the knowledge on post-transcriptional *var* gene regulation to the common *upsC* type. We identified a 101 nucleotide sequence that inhibits translation of *upsC*-derived mRNAs. Moreover, in the context of a heterologous upstream region, this 5` UTR element blocks reporter gene expression. In contrast to the repressive effect of the *upsE* element, however, *upsC*-mediated inhibition is irreversible. Interestingly, we found *var* 5` UTRs to be significantly enriched in uAUGs that are known to impair the efficiency of protein translation in other eukaryotes. Our findings suggest that regulation at the post-transcriptional level is a common feature in the control of PfEMP1 expression in *P. falciparum*.

3.2. Introduction

During intra-erythrocytic development, the human malaria parasite *Plasmodium falciparum* exports the major virulence factor erythrocyte membrane protein 1 (PfEMP1) to the red blood cell (RBC) surface[1]. The highly polymorphic N-terminal portion of PfEMP1 interacts specifically with a diverse set of endothelial host cell receptors such as CD36, ICAM1 or CSA[2–4]. Due to the adhesive properties of this integral membrane component, infected RBCs (iRBCs) disappear from peripheral circulation and thus avoid clearance in the

spleen[5]. The resulting aggregation of infected erythrocytes within the microvasculature of various organs is linked to severe forms of the disease such as cerebral or placental malaria[6].

In order to escape humoral immune responses *P. falciparum* employs antigenic variation of PfEMP1. The underlying mechanisms are based on a complex interplay of transcriptional and epigenetic control processes[7]. PfEMP1 is encoded by the multicopy *var* gene family whose members predominantly locate within subtelomeric domains, where they are interspersed by other species-specific virulence genes. In addition, some *var* genes occur in tandem clusters in central areas of some chromosomes[8]. Frequent recombination events generated a virtually limitless PfEMP1 sequence diversity that reflects the selective pressure acting on this immune-dominant antigen[9,10]. Notably, of the 60 *var* genes encoded in the haploid parasite genome, only a single variant is active at any given time[8,11]. This singular *var* gene choice is regulated at the level of RNA polymerase II-mediated transcription initiation and results in mutually exclusive expression of PfEMP1[12]. Each *var* gene represents a fully functional genomic unit that is associated with either of four 5` upstream (*ups*) regions. These conserved *upsA, B, C* and *E*-type regulatory sequences are equipped with *cis*-acting elements to control transcriptional activation and repression and the inclusion of each gene into the programme of singular *var* activity[8,13–16]. Several studies identified a central contribution of epigenetic mechanisms to the control of *var* gene transcription[15,17–19]. Subtelomeric and chromosome-internal *var* genes reside within heterochromatic, transcriptionally inert domains that cluster at the nuclear periphery[20–22]. The silenced and active states of *var* genes are earmarked by the differential occurrence of specific post-translational histone modifications. Most prominently, transcriptionally silenced *var* loci are associated with nucleosomes that harbour histone 3 tri-methylated at lysine 9 (H3K9me3)[23,24]. This specific histone modification recruits heterochromatin protein 1 (HP1)[24], a major component of transcriptionally silent chromatin[25]. The process of *var* gene activation occurs *in situ* and is accompanied by nuclear re-positioning of a formerly silenced locus into a transcriptionally competent perinuclear compartment[15,26]. In contrast to silenced loci, the active *var* gene is associated with H3K9 acetylation and H3K4me2/3 primarily in the *ups* region[17–19]. While in most of all cases daughter cells recapitulate the *var* transcription pattern of their progenitors due to epigenetic inheritance, occasional switching events result in antigenic variation of PfEMP1[11,19]. In line with the essential roles of histone modifying enzymes and effector proteins in this process, recent studies observed the partial or complete breakdown of singular *var* gene choice in response to interfering with histone de-

acetylation[18,27], H3K36 methylation[28] or PfHP1 recruitment (Brancucci *et al.*, unpublished data).

Generally, the molecular mechanisms regulating gene expression in *P. falciparum* are only poorly understood. Transcriptome profiling studies revealed that most genes, including the *var*s, exhibit a specific temporal activity pattern during the 48 hour intra-erythrocytic developmental cycle (IDC), suggesting that malaria parasites use gene-specific transcriptional activation and repression to produce transcripts only when their gene products are required[29–32]. However, in spite of similarities between the wave-like transcript and protein abundance profiles, crude mRNA and protein levels are only rarely in direct correlation[32–41], indicating that post-transcriptional mechanisms significantly contribute to the control of protein expression in *P. falciparum*. More specifically, according to mathematical models, the rates of mRNA translation and protein degradation account for most of the observed discrepancies[32].

In other life cycle stages, parasites make use of diverse strategies to store and reaccess pre-synthesised transcripts. The release of mRNA from translational repression in gametocytes and salivary gland sporozoites allows for a fast adaptation upon the change of host. Prior to gametocyte transmission, transcripts essential for ookinete formation are repressed and stabilised with the help of DOZI, a conserved DEAD-box RNA helicase[42,43]. At least for a subset these transcripts, translational repression is mediated by a conserved U-rich element found in either of the 5` or 3` untranslated region (UTR)[37,44]. Sporozoites employ a different mechanism to inhibit protein synthesis. Here, the phosphorylation of eukaryotic initiation factor 2α (eIF2α) by IK2, a serine/threonine protein kinase, results in a global suppression of translation and thus prevents cells in the salivary gland from pre-mature development into liver stage parasites[45].

Interestingly, the expression of a particular *var* gene, *var2csa*, was also found to be under specific post-transcriptional control[46–48]. VAR2CSA mediates adherence of iRBCs to chondroitin sulphate A (CSA) on placental syncytiotrophoblasts, which is responsible for pregnancy associated malaria[4,49–52]. *var2csa* expression is controlled by the unique *ups*E upstream sequence, and translation of the *var2csa* mRNA is reversibly repressed by the presence of a 360bp upstream open reading frame (uORF). This process is independent from expression of the uORF-encoded polypeptide[47] and translational re-initiation was recently reported as the rate-limiting step of VAR2CSA synthesis[48]. Whereas the 5` UTR affects translation efficiency also in house-keeping genes[53], other documented evidence for the involvement of post-transcriptional mechanisms in the control of *var* genes is lacking.

We recently identified a 101bp target sequence (MEE) in the upstream region of an *upsC var* gene that is essential for singular *var* gene choice[54]. Here, we show that in addition to its role on the chromatin level, the MEE sequence exerts a strong repressive effect on mRNA translation. Our data suggest that post-transcriptional regulation of *var* gene expression is a common mechanism in the control of mutually exclusive expression of PfEMP1.

3.3. Results

3.3.1. A *var* gene element inhibits heterologous promoter activity

The 101bp MEE element is located downstream of the transcriptional start site (TSS) in the *upsC* upstream region and controls inclusion of the locus into the programme of mutually exclusive *var* activity[54]. Here, we aimed at a more detailed functional characterisation of this regulatory sequence. First, we asked whether an *upsC* upstream sequence including the MEE is able to modulate gene expression autonomously when placed in a conserved position downstream of the TSS of a heterologous promoter. To achieve this, we used our previously published transfection vector pBK_{min} as a vehicle to target the endogenous *kahrp* (knob-associated histidine rich protein) locus[54]. pBK_{min} contains the blasticidin deaminase resistance gene followed by a reporter cassette where a minimal *kahrp* promoter (K_{min}) controls expression of the h*dhfr-gfp* (human dihydrofolate reductase fused to green fluorescent protein) reporter gene. Here, we replaced bps at position -445 to -1 with respect to the ATG start codon of the minimal *kahrp* promoter K_{min} with the *upsC* sequence (bps -519 to -1) containing the MEE (Figure 1A). Transfected 3D7 parasites were selected on blasticidin-S-HCl (BSD) and the plasmid was integrated into the endogenous *kahrp* locus by single-crossover homologous recombination (3D7/pBK_{min}C). This event created the *kahrp-upsC* hybrid upstream sequence *kahrp*C that drives expression of the h*dhfr-gfp* gene (Figure 1B). In this context, the wild-type *kahrp* promoter drives transcription of h*dhfr-gfp* and produces transcripts in which the 5` UTR of *kahrp* had been swapped with that of *var upsC* instead. Each of the downstream reporter cassettes on the integrated concatamer is flanked by the minimal $K_{min}$C 5` upstream region, whereas the endogenous *kahrp* gene is now controlled by the minimal K_{min} sequence. Note that these units are essentially inactive because K_{min} has negligible promoter activity[54].

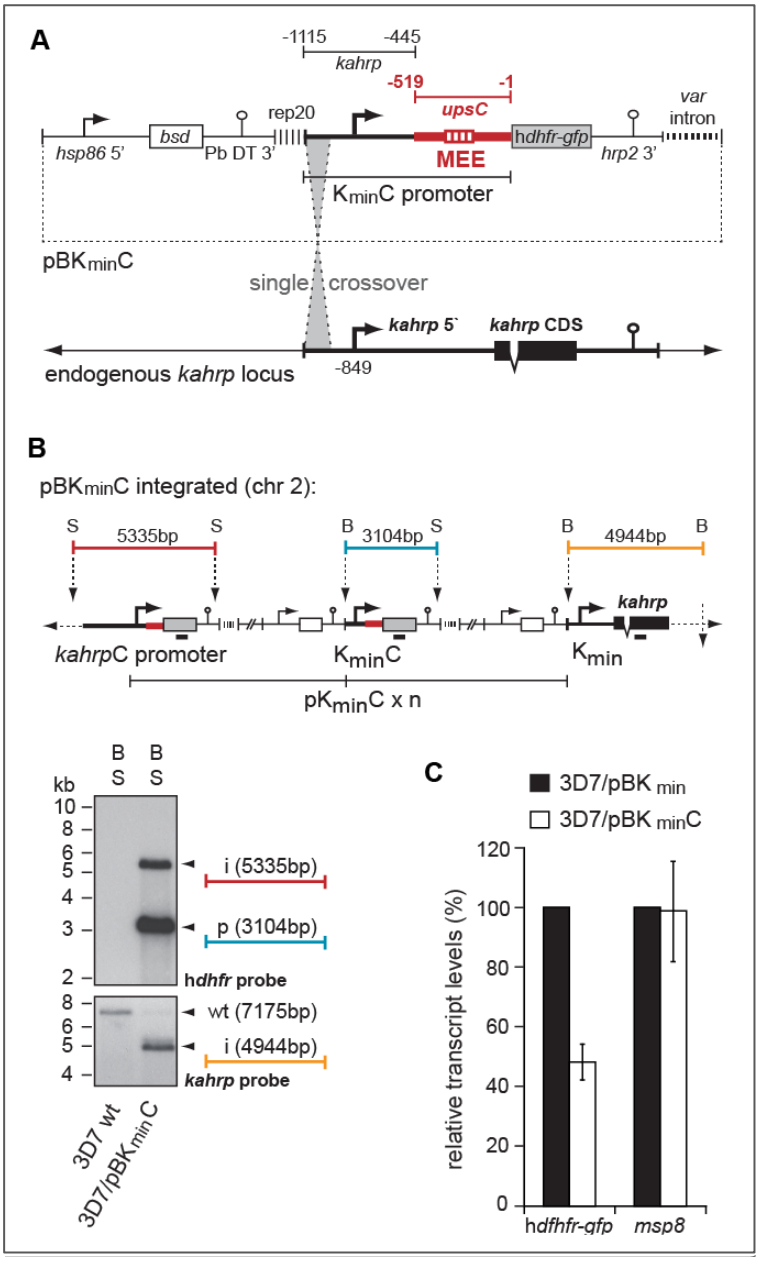


**Figure 1. Integration of the *upsC* 5` upstream sequence into a heterologous context at the *kahrp* locus.** (A) Schematic map of the transfection construct pBK$_{min}$C. Single-crossover integration was guided by *kahrp* 5` homology. The position of the *kahrp* TSS is indicated[55]. Numbers refer to the nucleotide positions relative to the ATG. The *bsd* resistance cassette selects for stably transfected parasites. The *var* intron is indicated by a bold dashed line. hsp86 5`, *hsp86* promoter; Pb DT 3`, *P. berghei dhfr*-thymidylate synthase terminator; rep20, 0.5kb TARE6 repeat element; hrp2 3`; histidine-rich protein 2 terminator. MEE, location of the 101bp mutual exclusion element MEE[54]. (B) Genomic situation after integration of the pBK$_{min}$C concatamer into the endogenous *kahrp* locus. Restriction sites used in Southern analysis and fragment lengths are indicated and colour-coded. S, *Stu*I; B, *Bgl*II. The Southern blot on *Bgl*II/*Stu*I-digested gDNA shows integration of pBK$_{min}$C into the endogenous locus of *kahrp*. The membrane was hybridised with h*dhfr* (top) and *kahrp* (bottom). Fragments are colour-coded according to the integration map. wt, size of the *kahrp* fragment in 3D7 wild-type parasites. i, integration event; p, plasmid fragment. (C) The *upsC* 5` UTR sequence represses *kahrp* promoter activity. The bars represent the ratio of relative h*dhfr-gfp* and *msp8* transcript levels in 3D7/pBK$_{min}$C parasites (black bars) compared to the 3D7/pBK$_{min}$ control (open bars). Results are the mean +/- s.d. of three independent experiments. Values are normalised for PF3D7_1331700 transcripts.

Surprisingly, 3D7/pBK$_{min}$C parasites were completely refractory to WR selection in several independent challenge experiments. To test if this was due to a block in transcription we performed quantitative reverse transcription-PCR (qRT-PCR) analysis. 3D7/pBK$_{min}$C parasites consistently displayed two-fold lower h*dhfr-gfp* transcript levels (52.0% +/- 17.3 s.d.) compared to control line 3D7/pBK$_{min}$[54] where the wild-type *kahrp* upstream sequence controls h*dhfr-gfp* transcription (Figure 1C). Albeit this result indicated that the *upsC* upstream sequence has a negative impact on *kahrp* promoter activity, a two-fold reduction in steady state transcript levels was unlikely to fully account for the irrevocable sensitivity of 3D7/pBK$_{min}$C to WR. Sequencing of reporter transcripts further excluded a scenario in which deleterious mutations may have been responsible for this prominent phenotype (data not shown).

3.3.2. The untranslated *var* element prevents translation of h*dhfr-gfp* reporter transcripts

In order to assess possible effects of the *upsC* 5` UTR on the post-transcriptional level we performed parallel semi-quantitative Northern and Western blot analyses (Figure 2). These experiments confirmed the reduced h*dhfr-gfp* transcript levels in 3D7/pBK$_{min}$C compared to the control line 3D7/pBK$_{min}$, which we already observed by qRT-PCR. As expected, *kahrp*C-derived transcripts had a slightly increased size compared to those originating from the wild-type promoter, demonstrating that the h*dhfr-gfp* mRNA was correctly transcribed in 3D7/pBK$_{min}$C. Strikingly, however, despite the presence of substantial amounts of steady state h*dhfr-gfp* transcripts, 3D7/pBK$_{min}$C parasites were unable to express the hDHFR-GFP protein (Figure 2). This result provides direct evidence for an important function of the *upsC* 5` UTR in translational inhibition and explains the refractoriness of 3D7/pBK$_{min}$C parasites to WR selection.

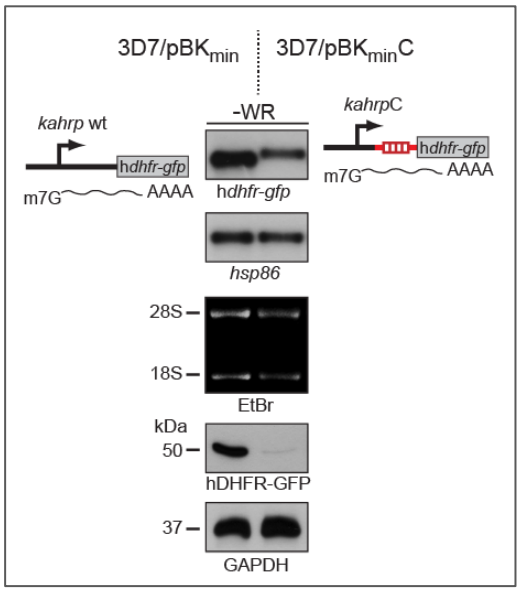


Figure 2. The *upsC* 5` UTR element inhibits translation. Semi-quantitative analysis of transcript and protein abundance in 3D7/pBKmin (control) and 3D7/pBKminC parasites cultured in absence of WR99210 (-WR). Top panels: h*dhfr-gfp* and *hsp86* (loading control) transcripts were detected by Northern blot. Ethidium bromide-stained 18S and 28S rRNAs serve as second loading control. Bottom panels: expression of hDHFR-GFP and GAPDH (loading control) in the same parasite samples were analysed by Western blot.

Noteworthy, we were eventually able to select for a WR-resistant 3D7/pBK$_{min}$C parasite population after twelve unsuccessful drug challenges. In light of these difficulties in generating a WR-resistant line, we considered a genomic rearrangement the most plausible cause for this altered phenotype. Indeed, Southern blot analysis revealed an additional h*dhfr-gfp* fragment in WR-selected compared to unselected parasites (Figure 3A). To determine this recombination event in exact detail, we carried out an elaborate mapping strategy based on further Southern blotting, ligation-mediated PCR and DNA sequencing (Figure S1 and S2). These efforts uncovered a major gene conversion event that resulted in the exchange of the end of chromosome 4 with a duplicated version of the end of chromosome 2 (Figure 3B). This occurred through the homologous recombination between a 10bp sequence directly upstream of the most telomere-proximal h*dhfr-gfp* gene on chromosome two and an identical 10bp sequence at the exon 1-intron boundary of *var* gene PF3D7_0400100 (FiguresS1 and S2). As a consequence, transcription of a single h*dhfr-gfp* gene in WR-resistant 3D7/pBK$_{min}$C parasites is now under control of the reverse strand of a *var* gene intron that was previously identified to possess bi-directional promoter activity[13]. Indeed, qRT-PCR using primers specific to this recombined locus and Western blotting identified active h*dhfr-gfp* transcription and hDHFR-GFP expression, respectively, in the WR-selected 3D7/pBK$_{min}$C population (Figures 3C and 3D). Northern blotting failed to detect intron-derived mRNA, which is explained by the relatively low abundance and the expected similar size of these transcripts compared to those derived from promoter K$_{min}$C. Furthermore, transcription at the PF3D7_0400100 intron was reported to initiate at variable sites[56], which additionally hampers detectability in Northern analysis. In line with the late peak activity of intron promoters during the IDC[56–58], synthesis of the novel h*dhfr-gfp* transcript species was induced following ring-stage development (Figure 3C). The emergence of this intron-derived mRNA in WR-selected cells confirms that the lack of hDHFR-GFP expression in unselected 3D7/pBK$_{min}$C parasites is solely caused by translational inhibition through the *upsC* 5` UTR. Moreover, the fact that bypassing this restriction was only possible through an extremely rare recombination event underscores the efficiency at which the *upsC* 5` UTR inhibits translation. In summary, we conclude that the *upsC* 5` upstream sequence investigated here exhibits a dual role in regulating expression; (i) as a DNA element it has a repressive effect on RNA PolII-dependent transcription, and (ii) as a 5` UTR element it efficiently prevents translation.

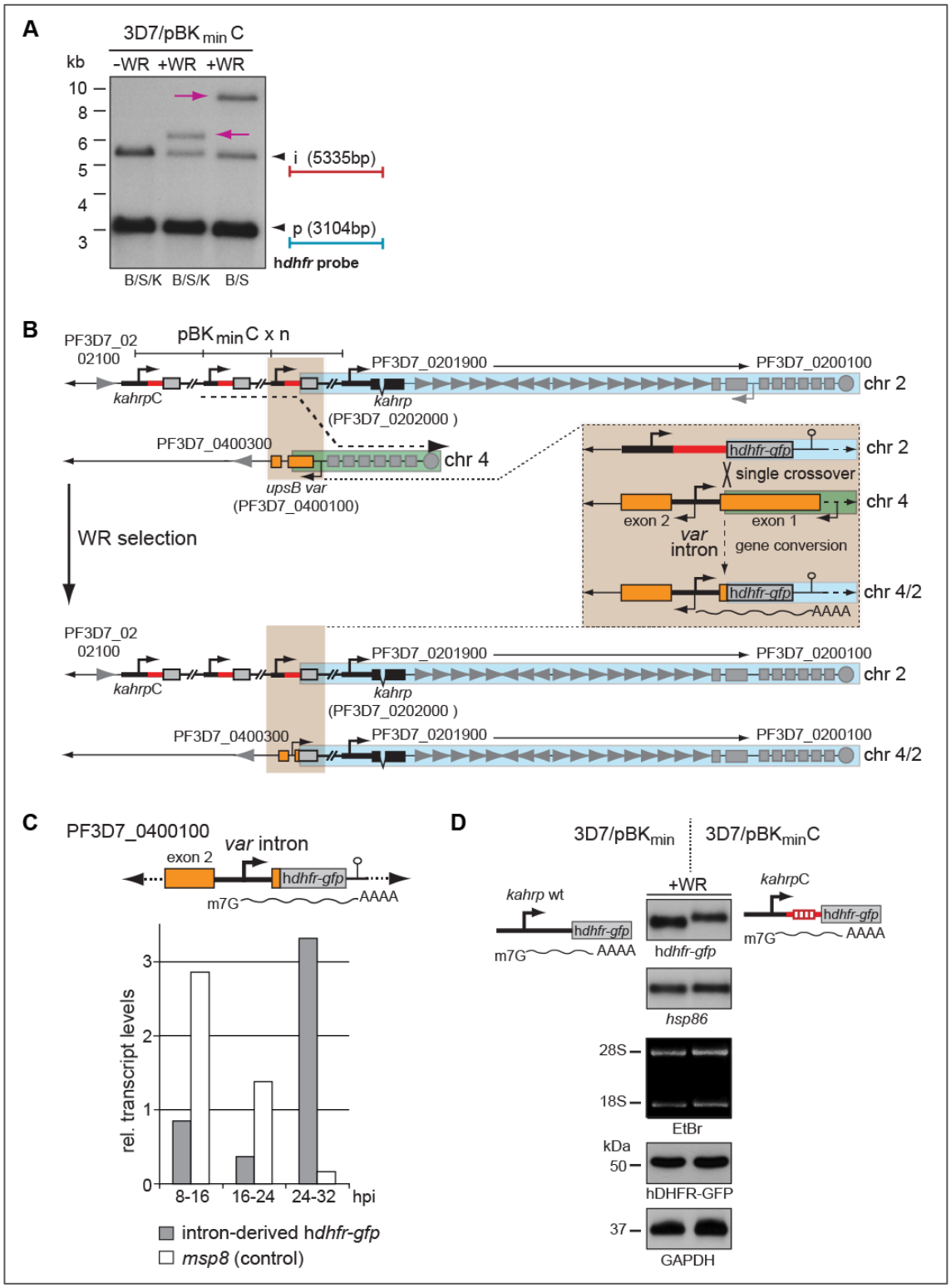


Figure 3. A gene conversion event revokes translational inhibition of h*dhfr-gfp* transcripts. (A) Southern analysis on digested gDNA from unselected and WR-selected 3D7/pBK$_{min}$C parasites. Additional h*dhfr*-containing fragments detected in WR-selected parasites only are highlighted by pink arrows. S, *Stu*I; B, *Bgl*II; K, *Kpn*I; i, integration event; p, plasmid fragment. (B) The ends of chromosome 2 and 4 in unselected and 4/2 in WR-selected parasites are schematically depicted. Gene IDs (www.plasmoDB.org) are indicated for a subset of genes as reference. The dashed arrow highlights the site of gene conversion. The blue box represents the duplicated region of chromosome 2. The green box represents the region of chromosome 4 that was deleted.

The brown box displays a zoom-in view of the gene conversion event and the resulting recombined locus. Detailed mapping and identification of the recombination site is presented in Figures S1 and S2. (C) h*dhfr-gfp* transcripts are produced from the *var* gene intron on chromosome 4 in WR-selected 3D7/pBK$_{min}$C parasites. Values represent relative *var* intron-derived h*dhfr-gfp* (black bars) and ring stage-specific *msp8* (open bars, control) transcript levels at three consecutive time points in WR-selected 3D7/pBK$_{min}$C parasites (normalised to PF3D7_1331700 transcripts). hpi, hours post invasion. (D) Semi-quantitative analysis of transcript and protein abundance in 3D7/pBK$_{min}$ (control) and 3D7/pBK$_{min}$C parasites cultured in presence of WR99210 (+WR). Top panels: h*dhfr-gfp* and *hsp86* (loading control) transcripts were detected by Northern blot. Ethidium bromide-stained 18S and 28S rRNAs serve as second loading control. Bottom panels: expression of hDHFR-GFP and GAPDH (loading control) in the same parasite samples were analysed by Western blot.

3.3.3. The MEE inhibits translation of *var* transcripts

Next, we tested if this *upsC* 5` UTR fragment also mediates translational inhibition in the natural context of *var* transcripts. To this end, we used a previously established set of transfected parasite lines[54] to investigate the effect of *upsC* 5` UTR truncations on steady state levels of both h*dhfr-gfp* reporter transcripts and the corresponding proteins using parallel qRT-PCR and semi-quantitative Western blot analyses (Figures 4A and 4B). Note that the introduced deletions have no effect on the temporal activity profile of the *upsC* promoter, not even in pBC4 where transcription is initiated from an alternative upstream TSS[54]. All cell lines investigated expressed similar amounts of hDHFR-GFP. However, 3D7/pBC4, 3D7/pBC5 and 3D7/pBC7 parasites displayed five- to over ten-fold lower total h*dhfr-gfp* transcript levels compared to 3D7/pBC and 3D7/pBC8,

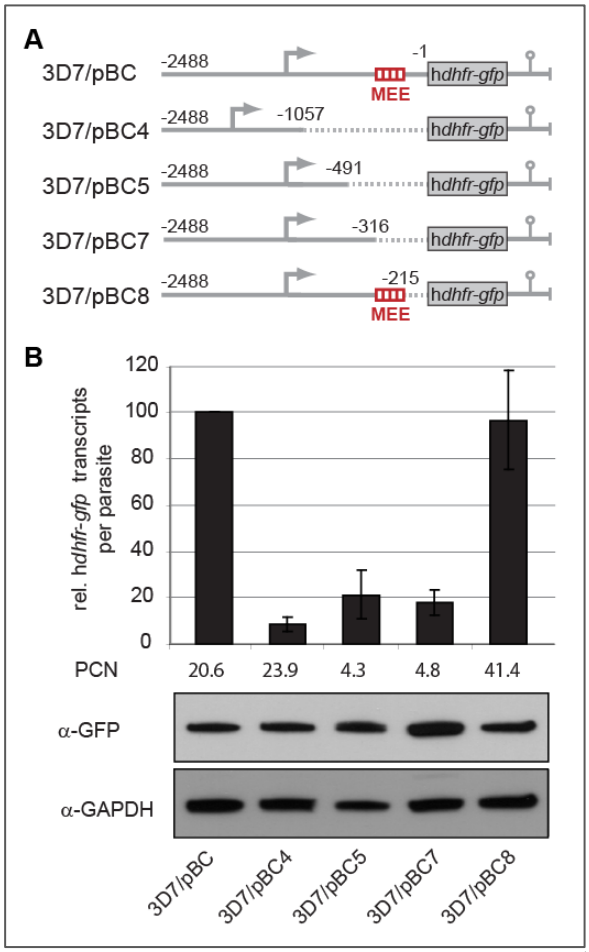


Figure 4. The MEE inhibits translation in the natural context of the *upsC* promoter. (A) Schematic depiction of *upsC var* promoter reporter constructs[54]. Deletions are represented by dashed lines. Numbers refer to the nucleotide positions relative to the ATG. (B) Proportion of steady-state h*dhfr-gfp* transcripts in parasites carrying truncated upstream sequences relative to the control line 3D7/pBC. Values are derived from three independent experiments (mean +/- s.d.) and represent total h*dhfr-gfp* transcripts per parasite (normalised to PF3D7_1331700 transcripts). Expression of hDHFR-GFP and GAPDH (loading control) in the same parasite samples was analysed by semi-quantitative Western blot. PCN, average plasmid copy numbers per parasite line.

demonstrating that these transcripts are translated with substantially higher efficiency (Figure 4B). Based on these results the inhibitory activity of the *upsC* 5` UTR can be assigned to the sequence located in between bps -316 to -215 with respect to the ATG start codon, which corresponds to the MEE element. These findings independently confirm the results obtained with the 3D7/pBK$_{min}$C line and further corroborate the critical function of the *upsC* 5` UTR in translational inhibition.

3.4. Discussion

Here, we describe the identification of an autonomous *cis*-acting element implicated in post-transcriptional *var* gene regulation. Insertion of bps -519 to -1 of the *upsC* 5` UTR into the context of the endogenous *kahrp* promoter rendered the corresponding hybrid transcripts incompetent for translation. Furthermore, the analysis of several truncated *upsC* sequences consistently showed that transcripts carrying a deletion of nucleotides -316 to -215 gave rise to significantly higher hDHFR-GFP protein levels compared to transcripts flanked by the wild-type 5` UTR. These combined results demonstrate that the *upsC* 5` UTR, or more precisely the MEE element, has a function in reducing the efficiency of translation.

The process of translation can be divided into initiation, elongation and termination. Among these phases, protein synthesis in eukaryotes is most highly regulated during initiation[59], i.e. the rate at which ribosomes launch proper genesis of the peptide chain. Usually, initiation is characterised by the recruitment of the translation pre-initiation complex (PIC) to the m7G cap at the 5` end of transcripts[60]. Once associated with mRNA, the PIC scans the untranslated region for downstream AUG start codons[61]. Both PIC recruitment and scanning can be impeded by secondary RNA structures, resulting in reduced initiation efficiency[62]. 5` polarity of the scanning mechanism provides further means to regulate translation as the first encountered start codon usually serves as a unique site of initiation[61]. Because of this “first AUG rule”, upstream start codons (uAUGs) can interfere with translation, often through creating small upstream open reading frames (uORFs). The encoded peptides, however, are only rarely involved in translational inhibition. In the case of *P. falciparum var2csa*, initiation at an uORF indeed prevents translation from the regular start codon in a reversible manner and it has been suggested that this process may allow for rapid switching to the VAR2CSA PfEMP1 variant under favouring environmental conditions[47]. The mechanisms underlying *upsC*-mediated translational inhibition identified in this study appear to be distinct from those operating in *var2csa* regulation. This hypothesis is

based on the observation that the inhibitory effect of the *upsC* 5` UTR is not reversible, demonstrating that inefficient translation is a hard-wired feature of *upsC*-derived transcripts. This is evident from the fact that the poor translation efficiency of *kahrp*C-derived h*dhfr-gfp* transcripts could never be reverted despite multiple attempts to select for WR-resistant 3D7/pBK$_{min}$C parasites. Hence, in contrast to the uORF in *var2csa*, the *upsC* element is unlikely to be involved in adaptive processes but rather fulfils gene-intrinsic post-transcriptional regulatory functions.

At this stage we do not know whether translation initiation at uAUGs and/or translation of uORFs is involved in the inhibitory function of the *upsC* 5` UTR. It is plausible that translational inhibition is mediated in a uORF-independent fashion, for instance by secondary mRNA structures and/or sequence specific RNA/protein interactions that may block PIC recruitment and/or scanning. Notably, however, we observed a prominent enrichment of uAUGs in *var* 5` UTRs in general compared to other ring stage-specific transcripts (Figure 5).

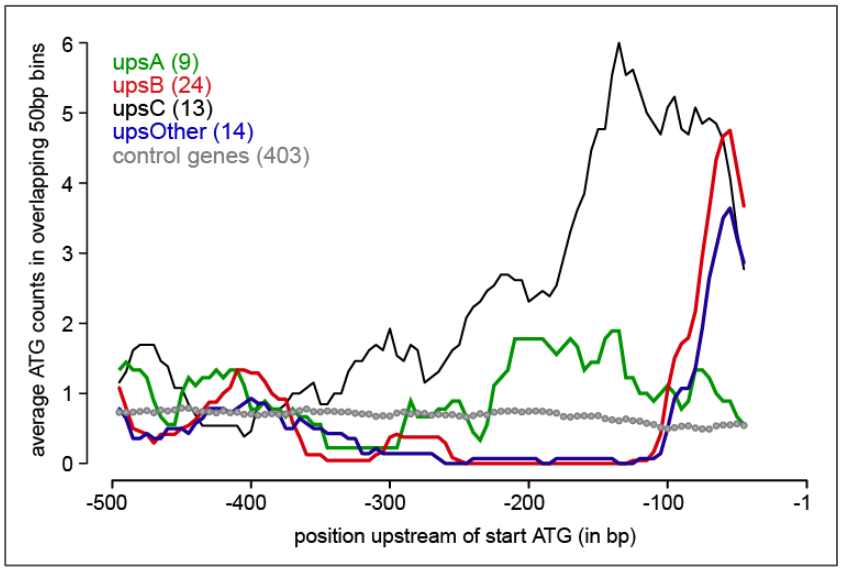


**Figure 5. uAUGs are enriched in *var* 5` UTRs**. For each gene, sequences ranging from bp -500 to -1 relative to the ATG start codon were downloaded from PlasmoDB version 7.2 (www.plasmoDB.org) and the counts of the trinucleotide sequence `ATG` were assessed in sliding windows of 50bps using custom-made perl scripts. The average ATG counts for each sequence set were plotted using the statistical analysis package R (www.r-project.org). The *var* gene set includes 60 sequences, subdivided into groups "*upsA*", "*upsB*", "*upsC*" and "others" (*upsB/C*, *upsB/A*, *upsE*) according to the classification by Lavstsen and colleagues[63]. The control set consists of 5` UTR sequences of 403 genes with peak transcription in ring stages. Selection of these sequences was based on RNA Seq data[31] according to the following criteria: timing of maximal expression: 8 hpi and 16 hpi; maximal expression ratio: 8-fold induction; maximum expression percentile: 30th percentile. uAUGs are significantly enriched in *var* 5` UTRs compared to the control set of ring stage-specific genes ($p=7.56x10^{-11}$; Welch t-test).

The investigated *upsC* sequence in $pBK_{min}C$ is no exception to that rule. In fact, the 519bp 5` UTR sequence contains a remarkable number of 33 uAUGs. Moreover, the 101bp MEE sequence element alone carries six uAUGs that may serve as initiation sites for the translation of 6-11aa long peptides. If uORF-translation plays a role in regulating expression of *var* genes other than *var2csa* remains to be investigated. Whereas the similar average size (4-6aa) of uORFs with a predicted function in yeast[64] supports such an assumption, conserved uORF-encoded peptides in *Drosophila* (70aa)[65] and the *var2csa* gene (120aa)[47] are much larger. Importantly, however, irrespective of whether translation is initiated within the *upsC* 5`UTR or not, uAUGs can lead to a substantial decrease in translation efficiency and they were shown to have important roles in translational control during development and conditions of cell stress[66,67]. Hence, *P. falciparum* must have evolved mechanisms to bypass the "first AUG rule" in order to express PfEMP1. This may be achieved through the well-known mechanisms of leaky uAUG scanning, re-initiation after uORF translation (as demonstrated for VAR2CSA expression[48]), or by using cap-independent strategies to guide ribosomes directly to the regular start site[61]. Although the exact mechanism by which translation of *upsC*-derived mRNA is inhibited remains to be determined, our findings demonstrate that *P. falciparum* uses this type of control to modulate expression of PfEMP1 variants. Similar to our observations, the 5` UTR of a house-keeping gene was recently identified to reduce translation efficiency[53], indicating that the observed phenomenon may apply to various classes of genes.

Because our experiments were based on the use of the h*dhfr-gfp* reporter system, it is important to mention that the hDHFR enzyme itself represses translation of its cognate mRNA by binding specifically to a 82bp element in the coding region[68–70]. Note that this effect occurs only in absence of specific inhibitors or substrates. In presence of inhibitors such as WR99210, the enzyme dissociates from the mRNA and translation efficiency increases[68,69,71]. This system of translational auto-regulation occurs not only in human cells but also in transgenic parasites expressing hDHFR[72]. It is therefore important to point out that this feedback mechanism fails to explain the different hDHFR-GFP translation efficiencies observed in our study. First, all parasite lines transfected with *ups*C constructs were constantly grown in presence of WR99210, i.e. under conditions where auto-inhibition of hDHFR expression is relieved. Even if some degree of auto-regulation remained, we would expect this to have occurred at equal levels in all lines. Second, translational inhibition of transcripts flanked by the *kahrp-upsC* hybrid 5` UTR was not reversible through treatment with WR99210 in multiple different experiments. Note that WR99210-induced release of

hDHFR auto-inhibition occurs rapidly within 24 hours after challenge[72] and that parasites expressing transcripts flanked by the wild-type *kahrp* 5` UTR were selected easily. Hence, if hDHFR-mediated auto-inhibition was responsible for our observations we would have expected rapid reversal of translational inhibition also for *kahrp*C- and *upsC*-derived transcripts.

What could be the function of this type of regulation in the control of *var* gene expression? The answer may lie in limitations of epigenetic mechanisms to strictly control singular expression of *var* genes. *var* transcription occurs through the escape of a single family member from transcriptionally inert heterochromatin that is associated with *var* loci. This process is linked to locus repositioning and the removal of local repressive epigenetic marks within a specialised perinuclear zone dedicated to *var* gene transcription[17–19,23,24,26,28]. It has also been speculated that a unique *trans-acting* DNA-sequence, similar to the H-element-mediated activation of mammalian olfactory receptor genes[73,74], may be involved in singular *var* gene choice[7]. However, recent efforts based on genome conformation capture technologies failed to detect such an element[75]. Irrespective of the exact mechanism underlying mutually exclusive *var* activation, silencing of all other *var* genes may not be entirely efficient. Indeed, there is evidence for the co-appearance of low levels of additional full-length *var* transcripts in individual parasites[76–78]. Hence, the repressive effect of *var* 5` UTRs may minimise the risk of translating low abundance *var* transcripts derived from incompletely silenced loci.

In summary, we show that the *upsC* 5` UTR autonomously mediates efficient translational inhibition. Our data are indicative for an involvement of upstream AUGs in this process, potentially leading to uORF expression. While beyond any doubt transcriptional and epigenetic control mechanisms dominate mutually exclusive *var* gene control, the strength of the observed effect indicates that translational inhibition may significantly contribute to prevent simultaneous expression of PfEMP1. In this context, it is interesting to note that both translational inhibition and mutually exclusive locus recognition are dependent on the MEE sequence element. It is therefore tempting to speculate that *P. falciparum* may have evolved a hard-wired control strategy that utilises a single regulatory element to control *var* gene expression at both the transcriptional and the translational level.

3.5. Materials and Methods

Parasite culture and transfection

P. falciparum 3D7 parasites were cultured as described previously[79]. Growth synchronisation was achieved by repeated sorbitol lysis[80]. Transfections were performed as described[15]. Parasites were selected on 2.5µg/ml blasticidin-S-HCl and 4nM WR99210. To obtain pBK$_{min}$C, the K$_{min}$ promoter in pBK$_{min}$[54] was replaced by a *Bgl*II/*Not*I-digested *kahrp* promoter fragment (-1115 to -445 bps) containing an additional *BamH*I restriction site at the 3` end directly upstream of the *NotI* site. The resulting plasmid was digested with *BamH*I/*Not*I to insert the *upsC* 5` UTR element (-519 to -1).

Western blot analysis.

Detection of hDHFR-GFP and GAPDH (loading control) was performed on whole cell lysates of parasites harvested at 6-14hpi. Primary antibody dilutions were: mouse anti-GFP (Roche Diagnostics, 11814460001), 1:500; monoclonal mouse anti-GAPDH 1-10B (kind gift of Claudia Daubenberger), 1:20`000.

Quantitative reverse transcription PCR

Pre-synchronised parasites cultures were synchronised twice 16 hours apart to obtain an eight-hour growth window. Total RNA was isolated using Tri Reagent (Ambion) and further purified using the RNeasy Plus Mini Kit (Qiagen) for removal of gDNA. Residual gDNA was digested with TURBO DNA-freeTM (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 of h*dhfr-gfp*, PF3D7_1331700 (glutamine-tRNA ligase), *msp8* and *var* intron-derived h*dhfr-gfp* were performed at final primer concentrations of 0.4µM using SYBR Green Master Mix (Applied Biosystems) on a StepOnePlusTM Real-Time PCR System (Applied Biosystems) in a reaction volume of 12µl. Plasmid copy numbers were determined by qPCR on gDNA isolated from the same parasite samples and calculated by dividing the absolute h*dhfr-gfp* copy numbers by the average value obtained for *msp8* and PF3D7_1331700. All reactions were run in duplicate yielding virtually identical Ct values. Serial dilutions of gDNA and plasmid DNA were used as standards for absolute quantification. Relative transcript values were

calculated by normalisation against the house-keeping gene PF3D7_1331700. Primer sequences are listed in Table S1.

Southern and Northern blot analysis

gDNA was digested with appropriate restriction enzymes overnight and separated on 0.5% TBE-buffered 0.7% agarose gels. Total RNA was isolated from saponin-released parasites using TriReagent (Ambion). RNA was glyoxylated for 1h at 55°C in five volumes glyoxal reaction mixture and electrophoresis was performed using 1xBPTE-buffered 1.5% agarose gels[81]. Blots were probed with ^{32}P-dATP-labeled h*dhfr*, *kahrp* and *hsp86* PCR fragments. Membranes were stripped by boiling in 0.1% SDS for 15min in between hybridisations.

3.6. References

1. Leech, J. H., Barnwell, J. W., Miller, L. H. & Howard, R. J. Identification of a strain-specific malarial antigen exposed on the surface of Plasmodium falciparum-infected erythrocytes. *J. Exp. Med.* **159,** 1567–1575 (1984).
2. Baruch, D. I., Gormely, J. A., Ma, C., Howard, R. J. & Pasloske, B. L. Plasmodium falciparum erythrocyte membrane protein 1 is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 3497–3502 (1996).
3. Rowe, J. A., Moulds, J. M., Newbold, C. I. & Miller, L. H. P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature* **388,** 292–295 (1997).
4. Reeder, J. C. *et al.* The adhesion of Plasmodium falciparum-infected erythrocytes to chondroitin sulfate A is mediated by P. falciparum erythrocyte membrane protein 1. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 5198–5202 (1999).
5. Langreth, S. G. & Peterson, E. Pathogenicity, stability, and immunogenicity of a knobless clone of Plasmodium falciparum in Colombian owl monkeys. *Infect. Immun.* **47,** 760–766 (1985).
6. Kyes, S., Horrocks, P. & Newbold, C. Antigenic variation at the infected red cell surface in malaria. *Annu. Rev. Microbiol.* **55,** 673–707 (2001).
7. Guizetti, J. & Scherf, A. Silence, activate, poise and switch! Mechanisms of antigenic variation in Plasmodium falciparum. *Cell. Microbiol.* **15,** 718–726 (2013).
8. Gardner, M. J. *et al.* Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature* **419,** 498–511 (2002).
9. Taylor, H. M., Kyes, S. A. & Newbold, C. I. Var gene diversity in Plasmodium falciparum is generated by frequent recombination events. *Mol. Biochem. Parasitol.* **110,** 391–397 (2000).
10. Ward, C. P., Clottey, G. T., Dorris, M., Ji, D. D. & Arnot, D. E. Analysis of Plasmodium falciparum PfEMP-1/var genes suggests that recombination rearranges constrained sequences. *Mol. Biochem. Parasitol.* **102,** 167–177 (1999).

11. Scherf, A. *et al.* Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum. *EMBO J.* **17,** 5418–5426 (1998).
12. Kyes, S. *et al.* Plasmodium falciparum var gene expression is developmentally controlled at the level of RNA polymerase II-mediated transcription initiation. *Mol. Microbiol.* **63,** 1237–1247 (2007).
13. Deitsch, K. W., Calderwood, M. S. & Wellems, T. E. Malaria. Cooperative silencing elements in var genes. *Nature* **412,** 875–876 (2001).
14. Dzikowski, R. *et al.* Mechanisms underlying mutually exclusive expression of virulence genes by malaria parasites. *EMBO Rep.* **8,** 959–965 (2007).
15. Voss, T. S. *et al.* A var gene promoter controls allelic exclusion of virulence genes in Plasmodium falciparum malaria. *Nature* **439,** 1004–1008 (2006).
16. Voss, T. S. *et al.* Alterations in local chromatin environment are involved in silencing and activation of subtelomeric var genes in Plasmodium falciparum. *Mol. Microbiol.* **66,** 139–150 (2007).
17. Lopez-Rubio, J. J. *et al.* 5' flanking region of var genes nucleate histone modification patterns linked to phenotypic inheritance of virulence traits in malaria parasites. *Mol. Microbiol.* **66,** 1296–1305 (2007).
18. Lopez-Rubio, J.-J., Mancio-Silva, L. & Scherf, A. Genome-wide analysis of heterochromatin associates clonally variant gene regulation with perinuclear repressive centers in malaria parasites. *Cell Host Microbe* **5,** 179–190 (2009).
19. Chookajorn, T. *et al.* Epigenetic memory at malaria virulence genes. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 899–902 (2007).
20. Marty, A. J. *et al.* Evidence that Plasmodium falciparum chromosome end clusters are cross-linked by protein and are the sites of both virulence gene silencing and activation. *Mol. Microbiol.* **62,** 72–83 (2006).
21. Freitas-Junior, L. H. *et al.* Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of P. falciparum. *Nature* **407,** 1018–1022 (2000).
22. Andrulis, E. D., Neiman, A. M., Zappulla, D. C. & Sternglanz, R. Perinuclear localization of chromatin facilitates transcriptional silencing. *Nature* **394,** 592–595 (1998).
23. Pérez-Toledo, K. *et al.* Plasmodium falciparum heterochromatin protein 1 binds to tri-methylated histone 3 lysine 9 and is linked to mutually exclusive expression of var genes. *Nucleic Acids Res.* **37,** 2596–2606 (2009).
24. Flueck, C. *et al.* Plasmodium falciparum heterochromatin protein 1 marks genomic loci linked to phenotypic variation of exported virulence factors. *PLoS Pathog.* **5,** e1000569 (2009).
25. Lomberk, G., Wallrath, L. & Urrutia, R. The Heterochromatin Protein 1 family. *Genome Biol.* **7,** 228 (2006).
26. Duraisingh, M. T. *et al.* Heterochromatin silencing and locus repositioning linked to regulation of virulence genes in Plasmodium falciparum. *Cell* **121,** 13–24 (2005).
27. Tonkin, C. J. *et al.* Sir2 paralogues cooperate to regulate virulence genes and antigenic variation in Plasmodium falciparum. *PLoS Biol.* **7,** e84 (2009).
28. Jiang, L. *et al.* PfSETvs methylation of histone H3K36 represses virulence genes in Plasmodium falciparum. *Nature* **499,** 223–227 (2013).
29. Bozdech, Z. *et al.* The transcriptome of the intraerythrocytic developmental cycle of Plasmodium falciparum. *PLoS Biol.* **1,** E5 (2003).
30. Llinás, M., Bozdech, Z., Wong, E. D., Adai, A. T. & DeRisi, J. L. Comparative whole genome transcriptome analysis of three Plasmodium falciparum strains. *Nucleic Acids Res.* **34,** 1166–1173 (2006).

31. Otto, T. D. *et al.* New insights into the blood-stage transcriptome of Plasmodium falciparum using RNA-Seq. *Mol. Microbiol.* **76,** 12–24 (2010).
32. Foth, B. J. *et al.* Quantitative time-course profiling of parasite and host cell proteins in the human malaria parasite Plasmodium falciparum. *Mol. Cell. Proteomics MCP* **10,** M110.006411 (2011).
33. Florens, L. *et al.* A proteomic view of the Plasmodium falciparum life cycle. *Nature* **419,** 520–526 (2002).
34. Lasonder, E. *et al.* Analysis of the Plasmodium falciparum proteome by high-accuracy mass spectrometry. *Nature* **419,** 537–542 (2002).
35. Le Roch, K. G. *et al.* Global analysis of transcript and protein levels across the Plasmodium falciparum life cycle. *Genome Res.* **14,** 2308–2318 (2004).
36. Gelhaus, C., Fritsch, J., Krause, E. & Leippe, M. Fractionation and identification of proteins by 2-DE and MS: towards a proteomic analysis of Plasmodium falciparum. *Proteomics* **5,** 4213–4222 (2005).
37. Hall, N. *et al.* A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses. *Science* **307,** 82–86 (2005).
38. Nirmalan, N., Sims, P. F. G. & Hyde, J. E. Quantitative proteomics of the human malaria parasite Plasmodium falciparum and its application to studies of development and inhibition. *Mol. Microbiol.* **52,** 1187–1199 (2004).
39. Tarun, A. S. *et al.* A combined transcriptome and proteome survey of malaria parasite liver stages. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 305–310 (2008).
40. Foth, B. J., Zhang, N., Mok, S., Preiser, P. R. & Bozdech, Z. Quantitative protein expression profiling reveals extensive post-transcriptional regulation and post-translational modifications in schizont-stage malaria parasites. *Genome Biol.* **9,** R177 (2008).
41. Briolant, S. *et al.* Plasmodium falciparum proteome changes in response to doxycycline treatment. *Malar. J.* **9,** 141 (2010).
42. Mair, G. R. *et al.* Regulation of sexual development of Plasmodium by translational repression. *Science* **313,** 667–669 (2006).
43. Mair, G. R. *et al.* Universal features of post-transcriptional gene regulation are critical for Plasmodium zygote development. *PLoS Pathog.* **6,** e1000767 (2010).
44. Braks, J. A. M., Mair, G. R., Franke-Fayard, B., Janse, C. J. & Waters, A. P. A conserved U-rich RNA region implicated in regulation of translation in Plasmodium female gametocytes. *Nucleic Acids Res.* **36,** 1176–1186 (2008).
45. Zhang, M. *et al.* The Plasmodium eukaryotic initiation factor-2alpha kinase IK2 controls the latency of sporozoites in the mosquito salivary glands. *J. Exp. Med.* **207,** 1465–1474 (2010).
46. Mok, B. W. *et al.* Default Pathway of var2csa switching and translational repression in Plasmodium falciparum. *PloS One* **3,** e1982 (2008).
47. Amulic, B., Salanti, A., Lavstsen, T., Nielsen, M. A. & Deitsch, K. W. An upstream open reading frame controls translation of var2csa, a gene implicated in placental malaria. *PLoS Pathog.* **5,** e1000256 (2009).
48. Bancells, C. & Deitsch, K. W. A molecular switch in the efficiency of translation reinitiation controls expression of var2csa, a gene implicated in pregnancy associated malaria. *Mol. Microbiol.* (2013). doi:10.1111/mmi.12379
49. Fried, M. & Duffy, P. E. Adherence of Plasmodium falciparum to chondroitin sulfate A in the human placenta. *Science* **272,** 1502–1504 (1996).
50. Salanti, A. *et al.* Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria. *Mol. Microbiol.* **49,** 179–191 (2003).

51. Salanti, A. *et al.* Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. *J. Exp. Med.* **200,** 1197–1203 (2004).
52. Montgomery, J. *et al.* Differential var gene expression in the organs of patients dying of falciparum malaria. *Mol. Microbiol.* **65,** 959–967 (2007).
53. Hasenkamp, S., Russell, K., Ullah, I. & Horrocks, P. Functional analysis of the 5' untranslated region of the phosphoglutamase 2 transcript in Plasmodium falciparum. *Acta Trop.* **127,** 69–74 (2013).
54. Brancucci, N. M. B., Witmer, K., Schmid, C. D., Flueck, C. & Voss, T. S. Identification of a cis-acting DNA-protein interaction implicated in singular var gene choice in Plasmodium falciparum. *Cell. Microbiol.* **14,** 1836–1848 (2012).
55. Lanzer, M., de Bruin, D. & Ravetch, J. V. A sequence element associated with the Plasmodium falciparum KAHRP gene is the site of developmentally regulated protein-DNA interactions. *Nucleic Acids Res.* **20,** 3051–3056 (1992).
56. Epp, C., Li, F., Howitt, C. A., Chookajorn, T. & Deitsch, K. W. Chromatin associated sense and antisense noncoding RNAs are transcribed from the var gene family of virulence genes of the malaria parasite Plasmodium falciparum. *RNA New York N* **15,** 116–127 (2009).
57. Calderwood, M. S., Gannoun-Zaki, L., Wellems, T. E. & Deitsch, K. W. Plasmodium falciparum var genes are regulated by two regions with separate promoters, one upstream of the coding region and a second within the intron. *J. Biol. Chem.* **278,** 34125–34132 (2003).
58. Kyes, S. A. *et al.* A well-conserved Plasmodium falciparum var gene shows an unusual stage-specific transcript pattern. *Mol. Microbiol.* **48,** 1339–1348 (2003).
59. Sonenberg, N. & Hinnebusch, A. G. Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets. *Cell* **136,** 731–745 (2009).
60. Pestova, T. V. *et al.* Molecular mechanisms of translation initiation in eukaryotes. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 7029–7036 (2001).
61. Kozak, M. Pushing the limits of the scanning mechanism for initiation of translation. *Gene* **299,** 1–34 (2002).
62. Kozak, M. Regulation of translation via mRNA structure in prokaryotes and eukaryotes. *Gene* **361,** 13–37 (2005).
63. Lavstsen, T., Salanti, A., Jensen, A. T. R., Arnot, D. E. & Theander, T. G. Sub-grouping of Plasmodium falciparum 3D7 var genes based on sequence analysis of coding and non-coding regions. *Malar. J.* **2,** 27 (2003).
64. Cvijović, M., Dalevi, D., Bilsland, E., Kemp, G. J. L. & Sunnerhagen, P. Identification of putative regulatory upstream ORFs in the yeast genome using heuristics and evolutionary conservation. *BMC Bioinformatics* **8,** 295 (2007).
65. Hayden, C. A. & Bosco, G. Comparative genomic analysis of novel conserved peptide upstream open reading frames in Drosophila melanogaster and other dipteran species. *BMC Genomics* **9,** 61 (2008).
66. Van der Velden, A. W. & Thomas, A. A. The role of the 5' untranslated region of an mRNA in translation regulation during development. *Int. J. Biochem. Cell Biol.* **31,** 87–106 (1999).
67. Spriggs, K. A., Bushell, M. & Willis, A. E. Translational regulation of gene expression during conditions of cell stress. *Mol. Cell* **40,** 228–237 (2010).
68. Chu, E., Takimoto, C. H., Voeller, D., Grem, J. L. & Allegra, C. J. Specific binding of human dihydrofolate reductase protein to dihydrofolate reductase messenger RNA in vitro. *Biochemistry (Mosc.)* **32,** 4756–4760 (1993).

69. Ercikan-Abali, E. A. *et al.* Dihydrofolate reductase protein inhibits its own translation by binding to dihydrofolate reductase mRNA sequences within the coding region. *Biochemistry (Mosc.)* **36,** 12317–12322 (1997).
70. Tai, N., Schmitz, J. C., Chen, T. & Chu, E. Characterization of a cis-acting regulatory element in the protein-coding region of human dihydrofolate reductase mRNA. *Biochem. J.* **378,** 999–1006 (2004).
71. Ercikan, E. *et al.* Translational regulation of the synthesis of dihydrofolate reductase. *Adv. Exp. Med. Biol.* **338,** 537–540 (1993).
72. Zhang, K. & Rathod, P. K. Divergent regulation of dihydrofolate reductase between malaria parasite and human host. *Science* **296,** 545–547 (2002).
73. Serizawa, S. *et al.* Negative feedback regulation ensures the one receptor-one olfactory neuron rule in mouse. *Science* **302,** 2088–2094 (2003).
74. Lomvardas, S. *et al.* Interchromosomal interactions and olfactory receptor choice. *Cell* **126,** 403–413 (2006).
75. Lemieux, J. E. *et al.* Genome-wide profiling of chromosome interactions in Plasmodium falciparum characterizes nuclear architecture and reconfigurations associated with antigenic variation. *Mol. Microbiol.* (2013). doi:10.1111/mmi.12381
76. Mok, B. W. *et al.* Comparative transcriptomal analysis of isogenic Plasmodium falciparum clones of distinct antigenic and adhesive phenotypes. *Mol. Biochem. Parasitol.* **151,** 184–192 (2007).
77. Noviyanti, R. *et al.* Multiple var gene transcripts are expressed in Plasmodium falciparum infected erythrocytes selected for adhesion. *Mol. Biochem. Parasitol.* **114,** 227–237 (2001).
78. Duffy, M. F. *et al.* Transcription of multiple var genes by individual, trophozoite-stage Plasmodium falciparum cells expressing a chondroitin sulphate A binding phenotype. *Mol. Microbiol.* **43,** 1285–1293 (2002).
79. Trager, W. & Jenson, J. B. Cultivation of malarial parasites. *Nature* **273,** 621–622 (1978).
80. Lambros, C. & Vanderberg, J. P. Synchronization of Plasmodium falciparum erythrocytic stages in culture. *J. Parasitol.* **65,** 418–420 (1979).
81. Sambrook, J. & Russell, D. W. Separation of RNA According to Size: Electrophoresis of Glyoxylated RNA through Agarose Gels. *CSH Protoc.* **2006,** (2006).

3.7. Supplementary Figure Legends

Figure S1. Confirmation of the gene conversion event by Southern blotting and ligation-mediated PCR. (A) The upper map schematically depicts the end of chromosome 2 including the integrated plasmid concatamer (blue box) in 3D7/pBK$_{min}$C parasites. *kahrp* promoter sequences are depicted by thick black lines. The *upsC* 5` UTR sequence is depicted in red. The grey circles and squares represent the telomeric tract and telomere-associated repeat elements (TAREs) 1-6, respectively. Arrowheads indicate ORFs. The gene accession number refers to the most telomere-proximal *upsB var* gene PF3D7_0200100. The lower map shows a zoom-in view of the integrated concatamer (blue box). Restriction sites used in Southern analysis are shown by vertical dashed arrows, and expected fragment lengths are indicated and colour-coded. The h*dhfr* probe used for hybridisation is shown below the h*dhfr-gfp* coding sequence (grey box). (B) The autoradiograph shows the hybridisation results obtained with the h*dhfr* probe after digesting 3D7/pBK$_{min}$C gDNA from unselected (-WR) and selected (+WR) populations with *EcoR*V/*Nco*I (red), *EcoR*V/*Spe*I (blue) or *EcoR*V/*Stu*I (green). Note the presence of an additional h*dhfr*-containing fragment after each double-digest specifically in WR-selected, but not in unselected parasites (highlighted by purple arrows). In each case, the size of the additional fragment (schematically depicted to the right, bottom) is approximately 2kb smaller than the size of the *EcoR*V/*Nco*I, *EcoR*V/*Spe*I or *EcoR*V/*Stu*I plasmid fragments (depicted to the right, top). This result suggested the presence of a novel *EcoR*V site upstream of a single copy of h*dhfr-gfp* (highlighted in purple). i, integration event; p, plasmid fragment. (C) Ligation-mediated PCR. gDNA from WR-selected 3D7/pBK$_{min}$C parasites was digested with *EcoR*V and *Nco*I and ligated into *EcoR*V/*Nco*I-digested pET-41 (EMD Biosciences). To amplify *EcoR*V/*Nco*I restriction fragments containing the h*dhfr* coding sequence, a primary PCR reaction was performed using T7 and h*dhfr*_R1 (R1) as forward and reverse primers, respectively. The primary PCR product was diluted 1:200 and used as template for a semi-nested PCR reaction using T7 and h*dhfr*_R2 (R2) as forward and reverse primers, respectively. This reaction yielded three specific amplicons each of which was sequenced using primer h*dhfr*_R3 (R3). Two fragments represented plasmid backbone sequences as a result of semi-specific amplification (data not shown). The nucleotide sequence of the third fragment is shown at the bottom (reversed sequence). It begins with an *EcoR*V site within the intron of *var* gene PF3D7_0400100 on chromosome 4 (orange letters) and continues into the 3` end of exon 1 (purple box). The green letters highlight the 10bp sequence involved in the recombination

event between the *var* and h*dhfr-gfp* loci. The grey box represents the start of the h*dhfr-gfp* coding sequence. A detailed schematic view of the recombination event is depicted above the nucleotide sequence. A single-crossover occurred between the 10bp sequence (green letters) directly upstream of the h*dhfr-gfp* reporter (grey box) on chromosome 2, and an identical sequence (green letters) at the very 3` end of exon 1 of *var* gene PF3D7_0400100 (purple box) on chromosome 4. As a result, the h*dhfr-gfp* reporter (grey box) was placed under control of the *var* gene intron promoter (orange line) on the reverse strand via gene conversion.

Figure S2. Further verification of the gene conversion event between chromosomes 2 and 4 in WR-selected 3D7/pBK$_{min}$C parasites. (A) The map schematically depicts the end of chromosome 2 including the integrated plasmid concatamer (blue box) in 3D7/pBK$_{min}$C parasites. *kahrp* promoter sequences are depicted by thick black lines. The *upsC* 5` UTR sequence is depicted in red. The grey circles and squares represent the telomeric tract and telomere-associated repeat elements (TAREs) 1-6, respectively. Arrowheads indicate ORFs. The gene accession number refers to the most telomere-proximal *upsB var* gene PF3D7_0200100. The lower map shows a zoom-in view of the integrated concatamer (blue box). Restriction sites used in Southern analysis are shown by vertical dashed arrows, and expected fragment lengths are indicated and colour-coded. The h*dhfr* probe used for hybridisation is shown below the h*dhfr-gfp* coding sequence (grey box). *Eco*RI sites are absent from the plasmid sequence. Hence, the *Eco*RI sites up- and downstream of the integrated concatamer release a restriction fragment in the size of 6228bps (chromosomal DNA) plus *n* times 9475bps (entire plasmid length) according to the number of copies in the concatamer. (B) The map schematically depicts the end of wild-type chromosome 4 including *var* gene PF3D7_0400100 (orange box) in unselected 3D7/pBK$_{min}$C parasites. The PF3D7_0400100 exon 1 probe used for hybridisation is shown below the coding sequence. The position of the *Eco*RI restriction site downstream of the *var* locus and the expected fragment length are indicated. (C) The map schematically depicts the end of chromosome 4 after the gene conversion event between chromosomes 2 and 4 in WR-selected 3D7/pBK$_{min}$C parasites ("chromosome 4/2 end"). The border between the green and blue boxes identifies the site of single-crossover recombination. The green and blue boxes represent sequences of the acceptor (chromosome 4) and donor (chromosome 2), respectively, of the gene conversion event. Restriction sites used in Southern analysis are shown by vertical dashed arrows, and expected fragment lengths are indicated and colour-coded. (D) The

autoradiograph shows the hybridisation results obtained after digesting 3D7/pBK$_{min}$C gDNA from unselected (-WR) and selected (+WR) populations with *EcoR*I, *EcoR*I/*Nco*I or *EcoR*I/*Sac*II. The membrane was hybridised with h*dhfr* (top) and PF3D7_0400100 (bottom) probes. Arrows are colour-coded according to the integration maps shown in panels A-C and identify the expected restriction fragments. The red, orange and yellow arrows highlight the restriction fragments that contain the single h*dhfr-gfp* cassette driven by the *var* intron promoter on chromosome 4/2 specifically in WR-selected parasites. Hybridisation with the PF3D7_0400100 exon 1 probe highlights the terminal chromosome 4 *EcoR*I fragment in unselected 3D7/pBK$_{min}$C parasites, which had been deleted from the genome in WR-selected parasites by the gene conversion event. i, integration event; p, plasmid fragment.

3.7.1. Supplementary Figures

Figure S1

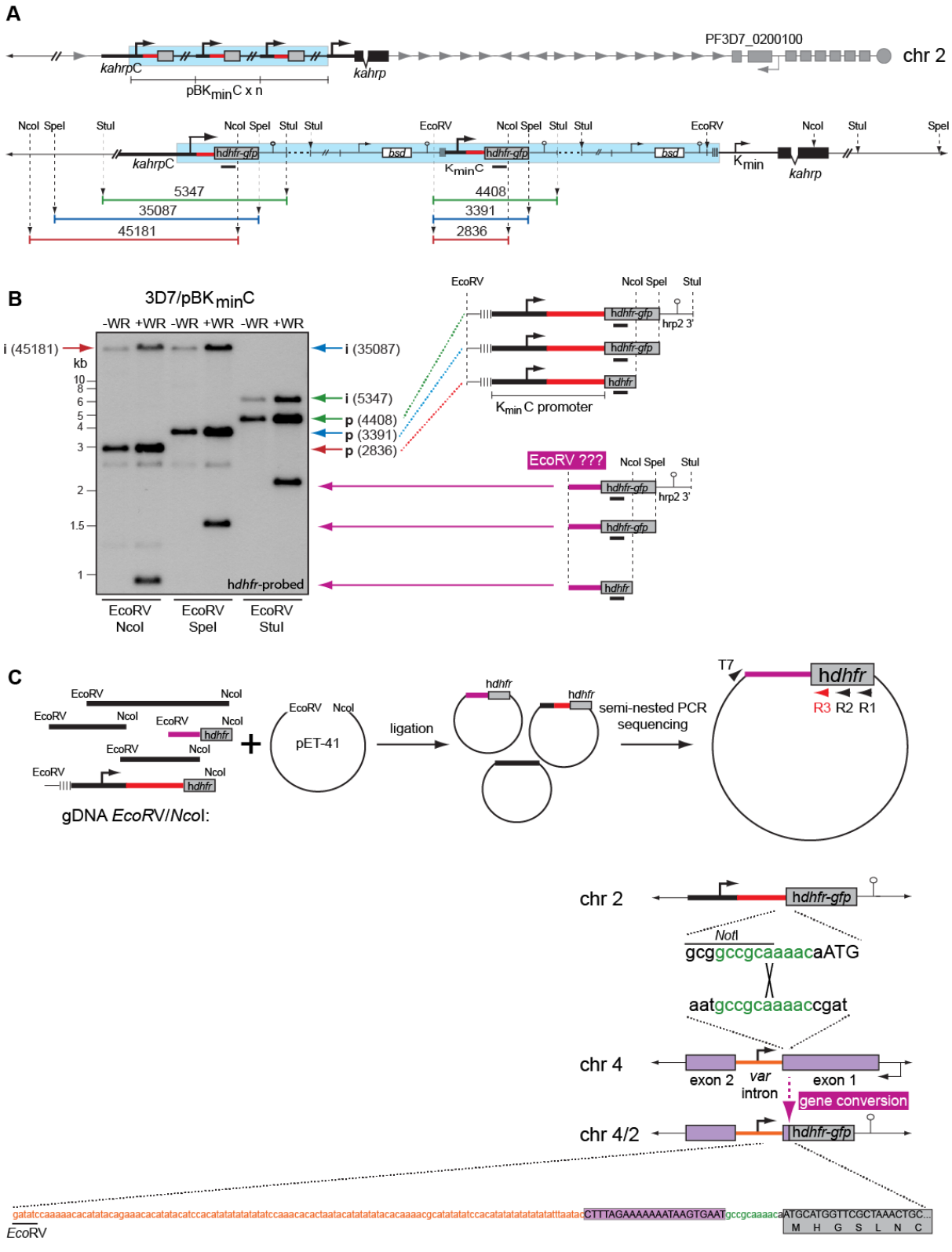
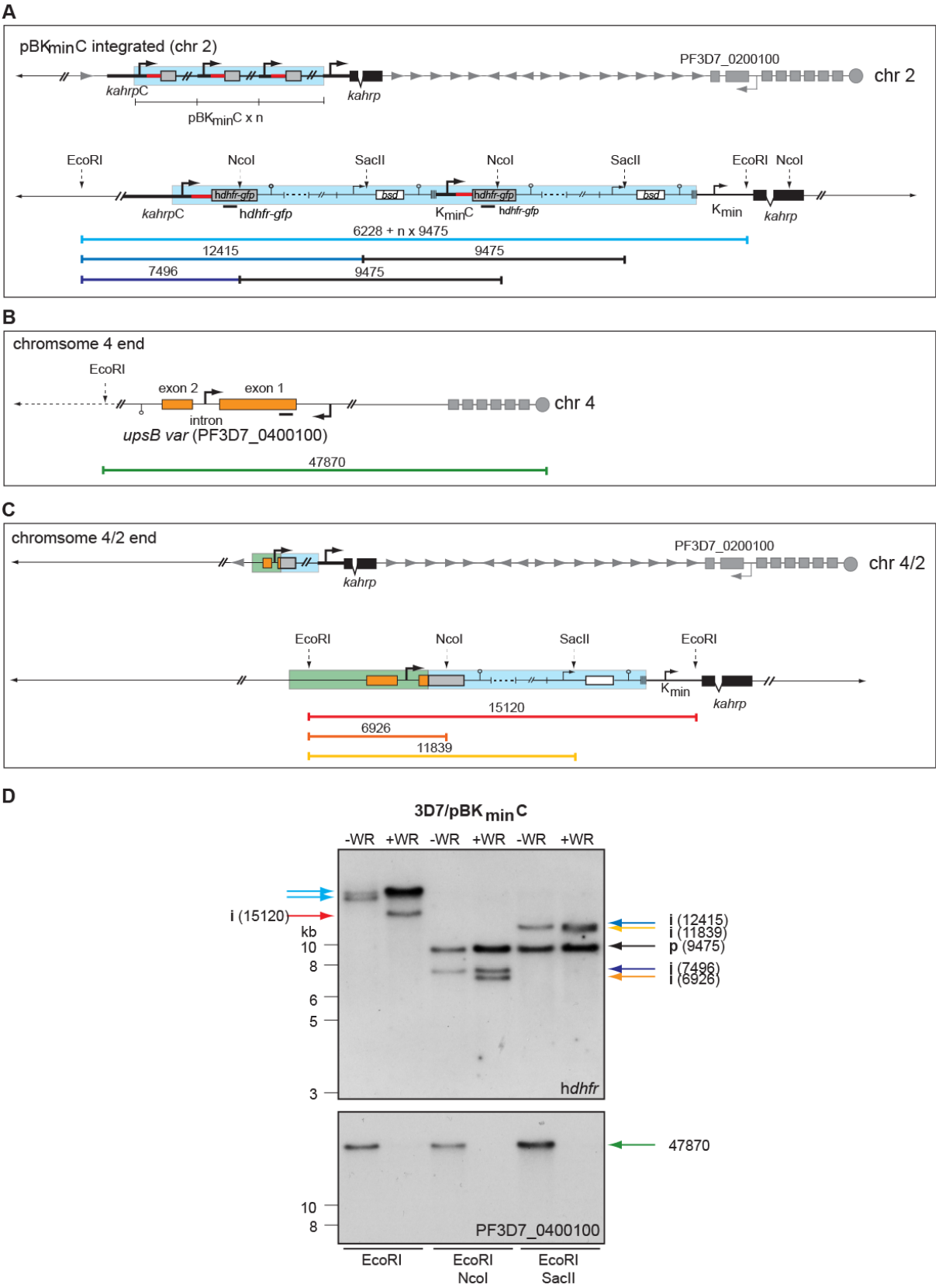


Figure S2


A
pBKminC integrated (chr 2)
PF3D7_0200100
chr 2
kahrpC
kahrp
pBKminC x n
EcoRI
NcoI
SacII
NcoI
SacII
EcoRI
NcoI
hdhfr-gfp
bsd
hdhfr-gfp
bsd
kahrpC
hdhfr-gfp
KminC
hdhfr-gfp
Kmin
kahrp
6228 + n x 9475
12415
9475
7496
9475
B
chromsome 4 end
EcoRI
exon 2
exon 1
intron
chr 4
upsB var (PF3D7_0400100)
47870
C
chromsome 4/2 end
PF3D7_0200100
chr 4/2
kahrp
EcoRI
NcoI
SacII
EcoRI
Kmin
kahrp
15120
6926
11839
D
3D7/pBKminC
-WR +WR -WR +WR -WR +WR
i (15120)
kb
10
8
6
5
3
hdhfr
i (12415)
i (11839)
p (9475)
i (7496)
i (6926)
47870
10
8
PF3D7_0400100
EcoRI
EcoRI
NcoI
EcoRI
SacII

Chapter 4

Heterochromatin protein 1 controls antigenic variation and sexual differentiation in malaria parasites

Nicolas M. B. Brancucci[1,2,4], Nicole L. Bertschi[1,2,4], Lei Zhu[3], Igor Niederwieser[1,2], Wai Hoe Chin[3], Rahel Wampfler[1,2], Céline Freymond[1,2], Matthias Rottmann[1,2], Ingrid Felger[1,2], Zbynek Bozdech[3], and Till S. Voss[1,2]*

[1]Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Socinstrasse 57, Basel 4051, Switzerland

[2]University of Basel, Petersplatz 1, Basel 4003, Switzerland

[3]School of Biological Sciences, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798, Singapore

[4]These authors contributed equally to this work

*Corresponding author: till.voss@unibas.ch; Phone: +41 61 284 81 61

4.1. Abstract

Heterochromatin protein 1 (HP1) is an important regulator of heterochromatin formation and epigenetic gene silencing in eukaryotes. Here, we conducted a comprehensive functional analysis of HP1 in the evolutionary divergent malaria parasite *Plasmodium falciparum*. We show that PfHP1 is indispensable for the heritable maintenance of mutually exclusive expression and antigenic variation of the major virulence factor PfEMP1. Furthermore, PfHP1 is essential for cell cycle progression at the G1/S transition phase. Most remarkably, PfHP1 controls the switch from vegetative growth to gametocyte differentiation by regulating the bistable expression of an ApiAP2 transcription factor. This important discovery establishes the first mechanistic details of the unidentified molecular pathway triggering sexual conversion. In summary, we uncovered fundamental roles for HP1-dependent epigenetic control processes for survival and transmission of malaria parasites. Our results provide unprecedented insight into the regulation of antigenic variation and sexual commitment, and reveal new opportunities for the development of intervention strategies blocking parasite proliferation and transmission.

4.2. Highlights

- Sexual commitment of malaria parasites is epigenetically controlled by HP1
- HP1 depletion disrupts singular *var* gene choice in *Plasmodium falciparum*
- HP1 is essential for mitotic proliferation of malaria blood stage parasites

4.3. Introduction

The protozoan parasite *Plasmodium falciparum* causes the most severe form of malaria in humans and is responsible for several hundred million clinical cases and 700’000 deaths annually (World Health Organisation, 2012). All malaria-associated morbidity and mortality is due to the blood stage of infection where successive rounds of intra-erythrocytic mitotic proliferation lead to the massive expansion of the parasite population. Extracellular merozoites rapidly invade red blood cells (RBCs) and mature intracellularly through the ring and trophozoite stage, before 4-5 iterative S/M phases produce a multinucleated schizont that eventually releases up to 32 merozoites ready to invade new RBCs. A small number of parasites exit the cell cycle and switch to sexual development through an unknown

mechanism. Once committed, these parasites differentiate over the next 8-10 days into mature male or female stage V gametocytes, the only stages capable of transmitting the infection to the mosquito vector (Baker, 2010).

To establish chronic blood stage infection and secure transmission, *P. falciparum* evolved a remarkable capacity to evade immune defence mechanisms through clonally variant gene expression (CVGE). The most striking example is erythrocyte membrane protein 1 (PfEMP1), the major antigen and prime immune target on the surface of infected RBCs (iRBCs) (Leech et al., 1984). 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). Importantly, *var* transcription obeys 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; Voss et al., 2006). 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). Other surface antigen families such as RIF (~150 members), STEVOR (~35) or PFMC-2TM (12) are also expressed in a clonally variant, albeit not mutually exclusive manner (Lavazec et al., 2007; Fernandez et al., 1999; Petter et al., 2007; Witmer et al., 2012).

The regulation of CVGE in *P. falciparum* is based on epigenetic mechanisms reminiscent of position-effect variegation (PEV) (Cortes et al., 2012). PEV describes the heritable and reversible silencing of genes located within or close to heterochromatin (Reuter and Spierer, 1992). Heterochromatin protein 1 (HP1), a major non-histone component of telomeric and peri-centromeric heterochromatin in eukaryotes (Lomberk et al., 2006b), plays a fundamental role in this process (Eissenberg et al., 1990). HP1 binds specifically to di-/tri-methylated lysine 9 of histone 3 (H3K9me2/3), the hallmark histone modification of heterochromatin, via its N-terminal chromodomain (CD) (Lachner et al., 2001). The C-terminal chromoshadow domain (CSD) mediates HP1 homodimerisation to facilitate chromatin condensation and recruits H3K9-specific methyltransferases (HKMTs) that modify adjacent nucleosomes (Cowieson et al., 2000; Aagaard et al., 1999; Yamamoto and Sonoda, 2003). As a result of these interactions, HP1 sustains a self-perpetuating mechanism for heterochromatin spreading and gene silencing along nucleosomal arrays.

In *P. falciparum*, HP1 binds to telomeres and telomere-associated repeat elements (TAREs) and to more than 400 genes that cluster in heterochromatic regions either directly

adjacent to TAREs or in some chromosome-internal domains (Flueck et al., 2009). The genome-wide PfHP1 occupancy pattern correlates strongly with the presence of H3K9me3 (Salcedo-Amaya et al., 2009; Lopez-Rubio et al., 2009). Remarkably, PfHP1-associated genes belong to over two dozen gene families that are specific to *P. falciparum* and subject to CVGE (Rovira-Graells et al., 2012). The vast majority of these genes encode antigens known or predicted to be exported to the iRBC surface (including *var*, *rifin*, *stevor* and *pfmc-2tm*) (Sargeant et al., 2006), while some are involved in erythrocyte invasion (*eba*, *rh*) (Cowman and Crabb, 2006) or nutrient uptake (*clag3*) (Nguitragool et al., 2011). In addition, PfHP1 binds to a small number of euchromatic loci including one gene coding for a putative ApiAP2 transcription factor (TF) (Balaji et al., 2005; Flueck et al., 2009). Presence or absence of PfHP1 or H3K9me3 at *var* and other PfHP1-associated loci correlates with silencing or activation, respectively (Chookajorn et al., 2007; Lopez-Rubio et al., 2007; Perez-Toledo et al., 2009; Jiang et al., 2010; Crowley et al., 2011; Coleman et al., 2012), and over-expression of PfHP1 leads to increased silencing of heterochromatic genes (Flueck et al., 2009). Together, these observations suggest key functions of 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 in these processes remains unknown.

Interestingly, the *pfhp1* locus is refractory to genetic deletion (Flueck et al., 2009; Perez-Toledo et al., 2009), which indicates additional vital roles for PfHP1 in parasite survival. Apart from heterochromatic gene silencing, HP1 proteins are involved in many other processes including chromosome segregation, telomere maintenance, regulation of euchromatic gene transcription, and DNA replication and repair (Hediger and Gasser, 2006; Kwon and Workman, 2008). This functional diversification is linked to the evolution of HP1 paralogs, particularly in opisthokonts, where HP1 isoforms display partially redundant but also distinct functions and genomic distributions (Lomberk et al., 2006b), and to the ability of HP1 to recruit a large number of functionally diverse effector proteins (Kwon and Workman, 2008). Unfortunately, in spite of the vast knowledge about HP1 function in model eukaryotes, the large evolutionary distance of *P. falciparum* precludes informed functional predictions for PfHP1. In contrast to model organisms, *P. falciparum* (i) encodes a single HP1 protein only; (ii) lacks obvious orthologs of many proteins known to interact with HP1; (iii) lacks the RNAi machinery (Baum et al., 2009) that is important for heterochromatic gene silencing in *S. pombe*, *D. melanogaster* and mammals (Pal-Bhadra et al., 2004; Hall et al., 2002;

Kanellopoulou et al., 2005); and (iv) does not display PfHP1/H3K9me3 enrichment in pericentromeric regions (Flueck et al., 2009).

Here, we conducted a comprehensive functional analysis of PfHP1 by generating a conditional PfHP1 loss-of-function mutant. We show that PfHP1 is essential for heterochromatic gene silencing and the maintenance of singular *var* gene choice. In addition, we discovered essential roles for PfHP1 in mitotic proliferation and gametocyte conversion. PfHP1 depletion prevents entry into S-phase and causes a reversible cell cycle arrest. Intriguingly, PfHP1 also controls the switch to sexual differentiation, and this is linked to the targeted de-repression of a euchromatic locus encoding an ApiAP2 transcription factor.

4.4. Results

4.4.1. PfHP1 is Indispensable for Mitotic Proliferation of Blood Stage Parasites

To investigate PfHP1 function we applied the FKBP destabilisation domain (DD) technique that allows modulating *in vivo* expression levels through the stabilising compound Shield-1 (Banaszynski et al., 2006; Armstrong and Goldberg, 2007). We generated a clonal parasite line in which endogenous PfHP1 is expressed as a C-terminally tagged GFP-DD fusion (Figure S1). In presence of Shield-1, 3D7/HP1^{ON} parasites exhibited no obvious growth phenotype and multiplied at a similar rate (3.8-fold +/-0.6 SD) as the control line 3D7/HP1^{ctrl} in which PfHP1 is tagged with GFP only (4.4-fold +/-0.4 SD) (Figure S1). After Shield-1 removal at 4-12 hours post-invasion (hpi), 3D7/HP1^{OFF} parasites progressed normally through the current intra-erythrocytic developmental cycle (IDC) and successfully re-invaded RBCs. In generation 2, however, 3D7/HP1^{OFF} parasites arrested at the early trophozoite stage (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} parasites (Figure 1B) (Flueck et al., 2009). By contrast, PfHP1 was undetectable in 3D7/HP1^{OFF} parasites 12 hours after Shield-1 withdrawal. A more direct assessment by parallel Western blot and IFA analyses showed that after Shield-1 removal at 4-12 hpi, PfHP1 expression was still detectable but reduced in late ring stages (16-24 hpi) and early schizonts (32-40 hpi) and the protein localised diffusely to the nucleo- and cytoplasm (Figure 1C and S2). After re-invasion, PfHP1 expression was undetectable in 3D7/HP1^{OFF} parasites by both IFA and Western blot. To test if PfHP1 was also depleted from chromatin

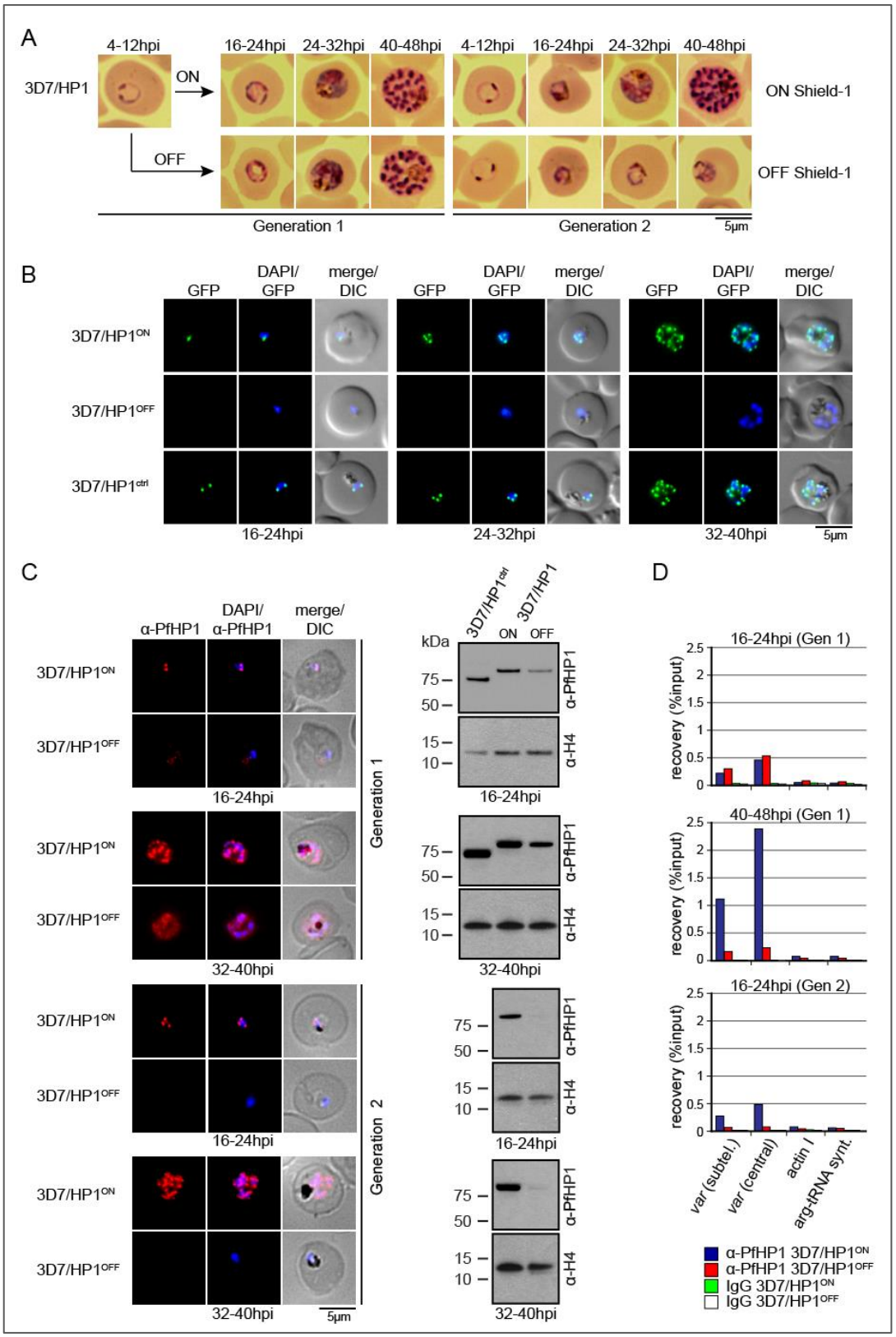


Figure 1. Growth phenotype of a conditional PfHP1 loss-of-function mutant and kinetics of PfHP1 depletion
(A) Morphological development of 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites over two generations (96 hrs). (B) Expression and localisation of PfHP1 by live cell microscopy. Images were taken at the indicated time points 12 hrs after Shield-1 removal. (C) Localisation and expression of PfHP1 by parallel IFA and Western blot analysis (Shield-1 removal at 4-12 hpi). (D) PfHP1 occupancy at two heterochromatic *var* and two euchromatic control loci (Shield-1 removal at 4-12 hpi).

we performed targeted chromatin immunoprecipitation (ChIP-qPCR) on two *var* loci located in either subtelomeric (PF3D7_0426000) or chromosome-internal (PF3D7_0412400) heterochromatin. After Shield-1 removal at 4-12 hpi, PfHP1-occupancy was unchanged in late ring stages but substantially reduced in schizonts and subsequent generation 2 ring stages (Figure 1D). Hence, Shield-1 removal does not deplete chromatin-associated PfHP1-GFP-DD in G1-phase. During schizogony, however, when PfHP1 is actively expressed, efficient degradation of PfHP1-GFP-DD prevents its incorporation into newly replicated chromatin.

To investigate the effect of PfHP1 depletion on parasite viability we performed 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 2A) (Wenzler et al., 2012). In generation 2, however, the metabolic activity changed dramatically in PfHP1-depleted parasites; heat emission remained low, but significantly higher compared to uninfected RBCs, over the entire 48 hour period of measurement. Interestingly, single cell DNA content analysis revealed that these parasites failed to undergo genome replication and were apparently blocked at the G1/S transition phase (Figure 2B).

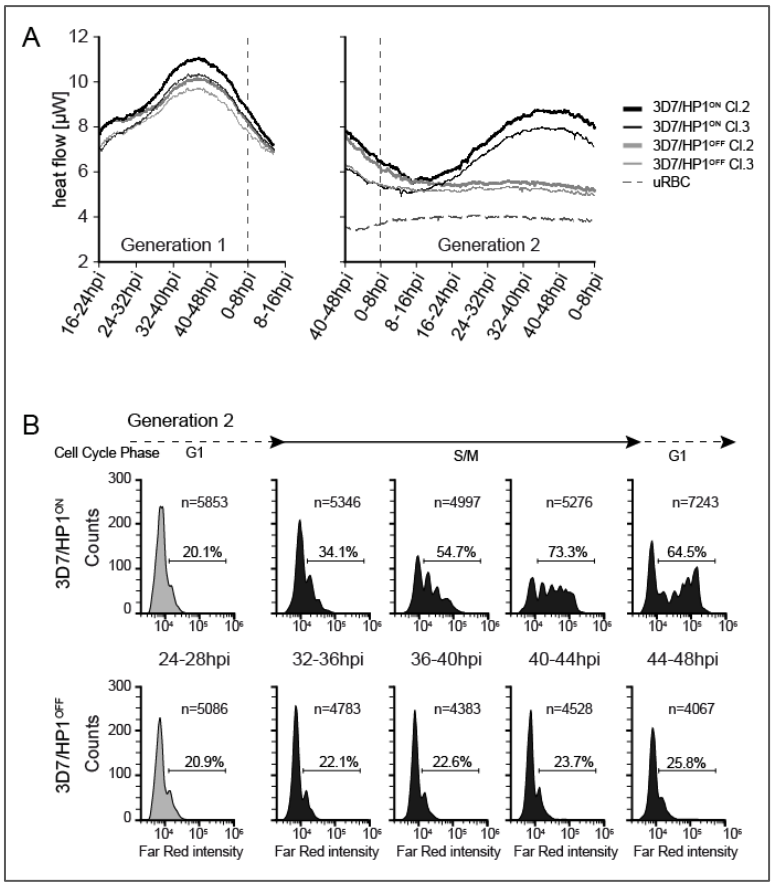


Figure 2. PfHP1 depletion causes cell cycle arrest prior to S-phase
(A) Heat emission in two 3D7/HP1^{ON} clones (Cl.2 and Cl.3) grown in presence or absence of Shield-1. The vertical dashed lines indicate the time point of re-invasion. uRBC, uninfected RBCs.
(B) Flow cytometry histograms showing DNA content in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites at five consecutive time points in generation 2. The percentage of iRBCs with >2 parasite genomes is indicated. Prior to S-phase (grey) this value corresponds to the proportion of RBCs infected with >2 parasites (confirmed by microscopy).

In summary, these data show that PfHP1 is essential for mitotic proliferation. In absence of PfHP1 parasites remain viable but fail to enter or progress through S-phase. Notably, functional complementation with PfHP1-CherryFP fully compensated for the loss of PfHP1-GFP-DD expression, which unambiguously proves the causal relationship between PfHP1 depletion and the pronounced growth phenotype observed (Figure S3).

4.4.2. PfHP1 Controls Sexual Differentiation

Prolonged observation of PfHP1-depleted parasites revealed an intriguing transition into two phenotypically distinct subpopulations; G1-stalled asexual parasites and sexual forms undergoing gametocyte development (Figure 3A).

To discriminate quantitatively between these forms, we performed double-labelling IFAs using antibodies against Pfs16 (a gametocyte-specific marker expressed in stage I-V gametocytes) (Bruce et al., 1994) and knob-associated histidine-rich protein (KAHRP) (a marker for iRBCs) (Taylor et al., 1987). Strikingly, 52.7% (+/-3.1 SD) of 3D7/HP1^{OFF} parasites expressed Pfs16 at 32-40 hours post re-invasion, compared to only 2.3% (+/-1.2 SD) in the 3D7/HP1^{ON} population (Figures 3B and 3C). This remarkable sexual conversion rate was confirmed 24 hours later when parasites already displayed stage II-specific morphology (Figure 3B). Notably, PfHP1-depleted gametocytes completed sexual development within 8-10 days with similar dynamics and morphology as 3D7/HP1^{ctrl} gametocytes (Figure 3D). These data show that PfHP1 controls cell fate decision of blood stage parasites by antagonising sexual differentiation. Depletion of PfHP1 leads to the synchronous hyper-induction of viable gametocytes able to complete sexual development independent of PfHP1.

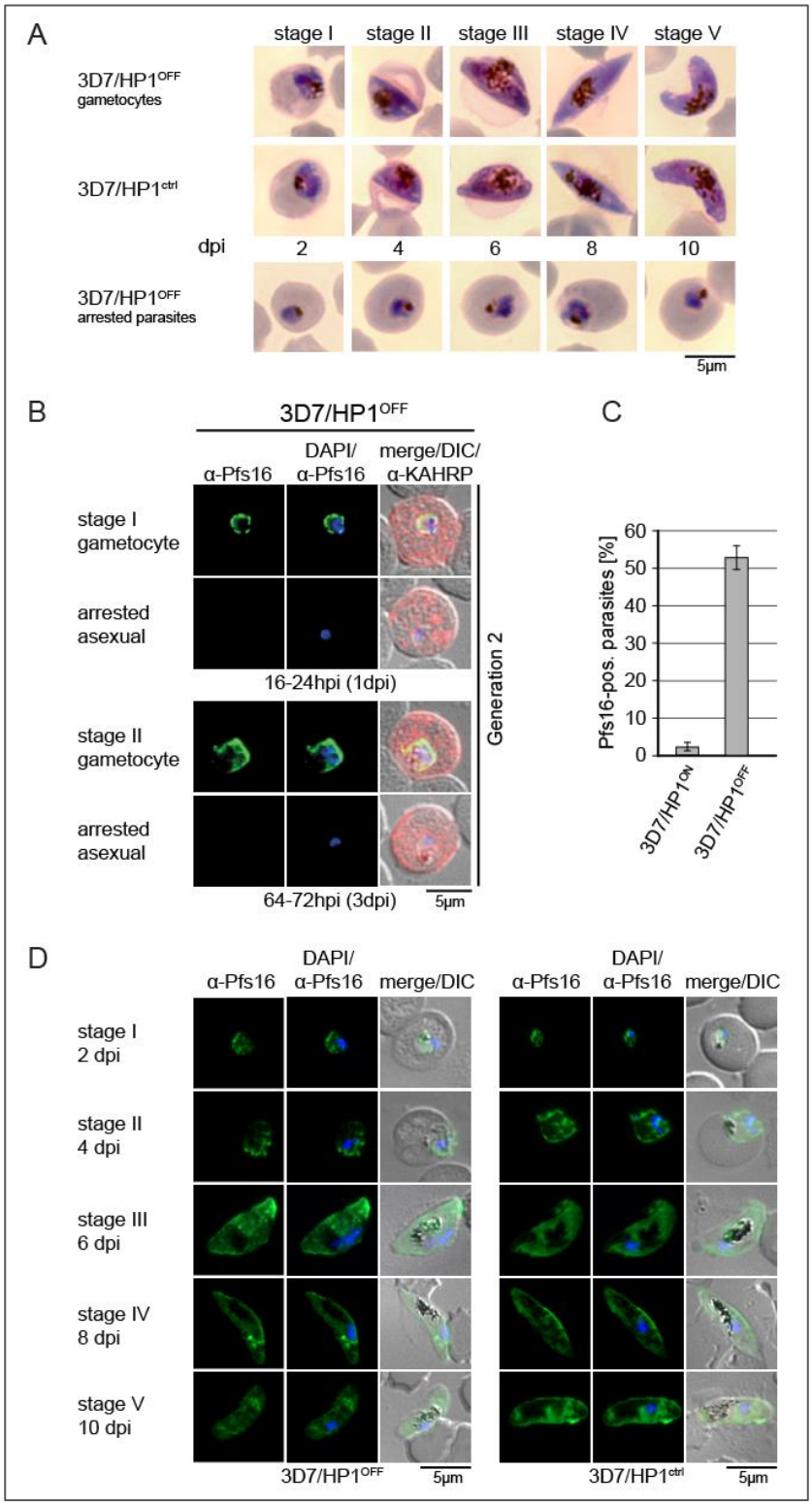


Figure 3. PfHP1 depletion induces gametocyte conversion

(A) 3D7/HP1^{OFF} parasites develop into gametocytes or undergo cell cycle arrest. (B) IFAs distinguish between 3D7/HP1^{OFF} early gametocytes and arrested asexual trophozoites. (C) Proportion of Pfs16-positive 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites as determined by IFA at 32-40 hours post re-invasion. Values show the mean of three biological replicates (+/- SD) (>100 parasites analysed in each experiment). (D) Morphology and development of 3D7/HP1^{OFF} and 3D7/HP1^{ctrl} stage I-V gametocytes by IFA. dpi, days post re-invasion.

4.4.3. PfHP1-Depleted Asexual Parasites Enter a Reversible Cell Cycle Arrest

Interestingly, cell cycle-arrested 3D7/PfHP1^{OFF} trophozoites were able to re-enter mitotic proliferation when Shield-1 was replenished (Figure 4A). Even after 12 days in absence of Shield-1, rescued trophozoites re-accumulated perinuclear PfHP1 and progressed through schizogony (Figure 4B). 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). We also observed a gradual decrease in parasitaemia and increase in time required for growth resumption after replenishing Shield-1, showing that increasing numbers of PfHP1-depleted parasites died over time (Figures 4A and S4). These data corroborate the essential function of PfHP1 for mitotic proliferation and show that a subset of PfHP1-depleted trophozoites remains in a state of dormancy capable of re-entering the cell cycle if PfHP1 expression is restored.

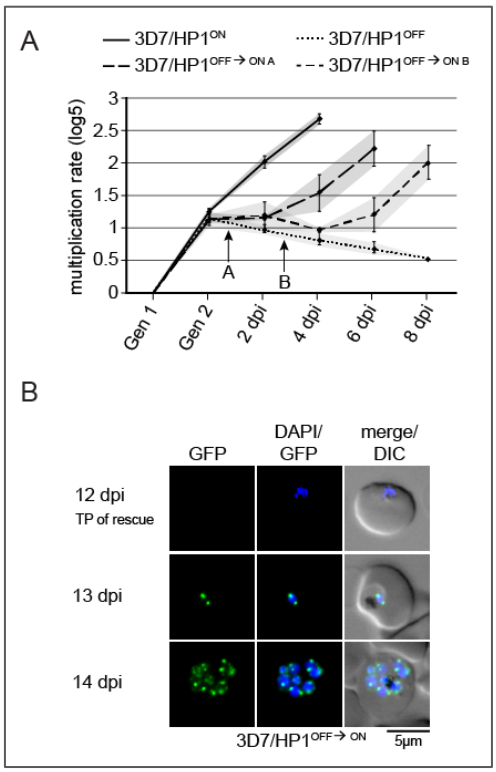


Figure 4. Cell cycle-arrested trophozoites remain viable and can re-enter mitotic proliferation
(A) Cell cycle-arrested 3D7/HP1^{OFF} parasites re-establish asexual growth after adding back Shield-1 at 24 ("A") or 72 ("B") hours post re-invasion (arrows). Values show the mean of three biological replicates (+/- SD). (B) 3D7/HP1^{OFF} parasites re-accumulate PfHP1 and re-enter mitotic proliferation after re-addition of Shield-1 at 12 dpi. dpi, days post re-invasion.

4.4.4. Lack of S/M-Phase Entry Correlates with Decelerated Transcriptome Progression in G1-Phase

We next performed comparative transcriptome profiling to study the effect of PfHP1 depletion on parasite proliferation, heterochromatic gene silencing and gametocyte conversion. Note that most *P. falciparum* genes follow a tightly controlled temporal pattern of transcriptional activity during the 48-hour IDC (Bozdech et al., 2003). Hence, to identify dysregulated genes with high confidence we harvested total RNA from paired synchronous 3D7/HP1^{ON} and 3D7/HP1^{OFF} samples at eleven consecutive time points (TPs) spanning two generations for genome-wide microarray analysis (Figure 5A and Table S1).

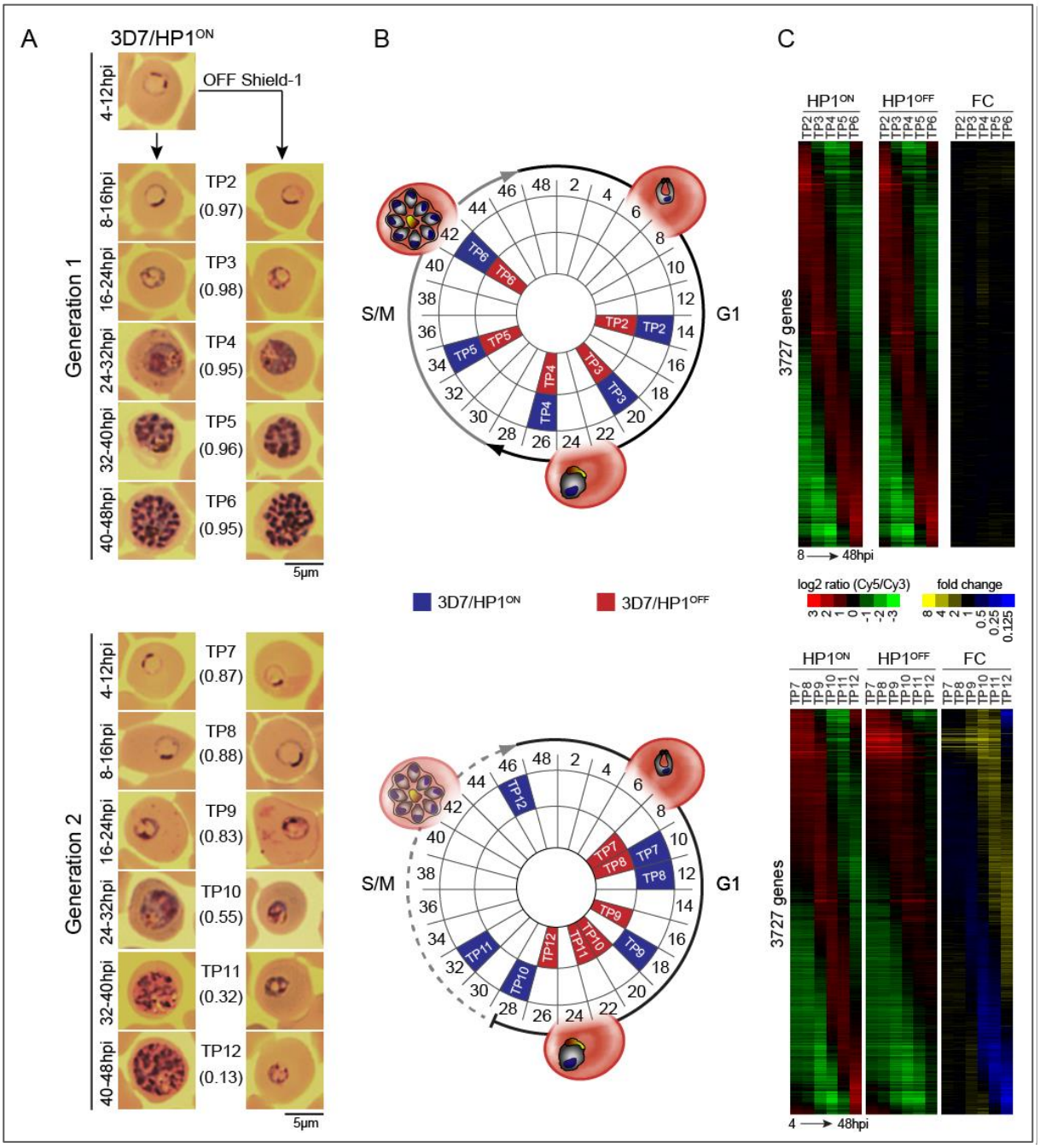


Figure 5. PfHP1-depletion leads to a marked slowdown in transcriptome progression.
(A) 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites were sampled for comparative transcriptome analysis. Similarity between transcriptome pairs is indicated in by Pearson correlation coefficients (bracketed). (B) Mapping of experimental transcriptomes to a high-resolution reference dataset. Blue and red boxes identify the best-fit reference TP (hpi) for each 3D7/HP1^{ON} and 3D7/HP1^{OFF} transcriptome, respectively. Spearman rank coefficients are provided in Table S2. (C) Comparison of global temporal expression profiles between 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites. Heat maps display relative gene expression levels (red/green) and fold changes (FC) in gene expression (yellow/blue). Heat maps are ordered according to the phase calculated for 3D7/HP1^{ON} parasites (TPs7-12), starting at $-\pi/2$ and include 3727 genes (sine-fit p-value <0.01).

Until 16-24 hpi in generation 2 (TP9), the transcriptomes were highly similar between both populations (r=0.82-0.98) (Figure 5A). A Spearman-rank correlation method mapping each experimental transcriptome to the best-fit time point in a high-resolution reference IDC dataset (Mok et al., 2011; Foth et al., 2011) showed further that both lines progressed with similar kinetics through the first IDC and second generation ring stage development (Figure 5B and Table S2). No major changes in temporal gene activity or relative expression were obvious in generation 1 (Figure 5C). In contrast, at 24-48 hours after re-invasion (TPs 10-12), when the 3D7/HP1^{OFF} population consisted of a mixture of early gametocytes and arrested trophozoites, the transcriptomes correlated poorly (r=0.13-0.55) (Figure 5A). The slowdown in transcriptome development in 3D7/HP1^{OFF} parasites reflects a substantial deceleration in G1 progression and failure to enter S-phase (Figure 5B). This is further emphasised by the massive accumulation of ring stage- and trophozoite-specific transcripts and lack of activation of schizont-specific genes (Figure 5C). Based on this initial assessment we selected TPs 4-9 for further comparative analysis to identify genes differentially expressed in response to PfHP1 depletion.

4.4.5. PfHP1 Silences Heterochromatic Genes and is Essential for the Maintenance of Singular *var* Gene Choice

Comparison of mean relative transcript abundances revealed de-repression of heterochromatic genes in 3D7/HP1^{OFF} parasites with 111 genes (32%) displaying a >1.5-fold increase in expression (fdr<0.1) (Figure 6A 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. Even among the non-significantly de-regulated heterochromatic genes the majority was still up-regulated in absence of PfHP1 (Figure S5).

We next investigated the prevailing role of PfHP1 in *var* gene silencing in more detail. Most parasites in the 3D7/HP1^{ON} clone expressed *var2csa* (PF3D7_1200600) (Salanti et al., 2003) and a few smaller subpopulations, in which switching to a different *var* gene had occurred, were also evident (Figures 6B and S6). Removal of Shield-1 at 4-12 hpi had no immediate effect on *var* transcription, 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, the entire *var* repertoire was massively up-regulated after re-invasion and individual genes showed up to 30-fold higher expression levels. Importantly, transcription of the few *var* genes that were already dominantly expressed in 3D7/HP1^{ON} parasites was unchanged or slightly elevated only (Figures 6B and S6). This shows that the de-repression of all other *var* genes occurred in parallel to, rather than at the expense of, the transcription of the previously active gene. These results were also reflected at the protein level. 3D7/HP1^{ON} parasites predominantly expressed a single large PfEMP1 variant consistent with the size of VAR2CSA, whereas PfHP1-depleted parasites expressed several variants of different sizes in addition to VAR2CSA (Figure 6C).

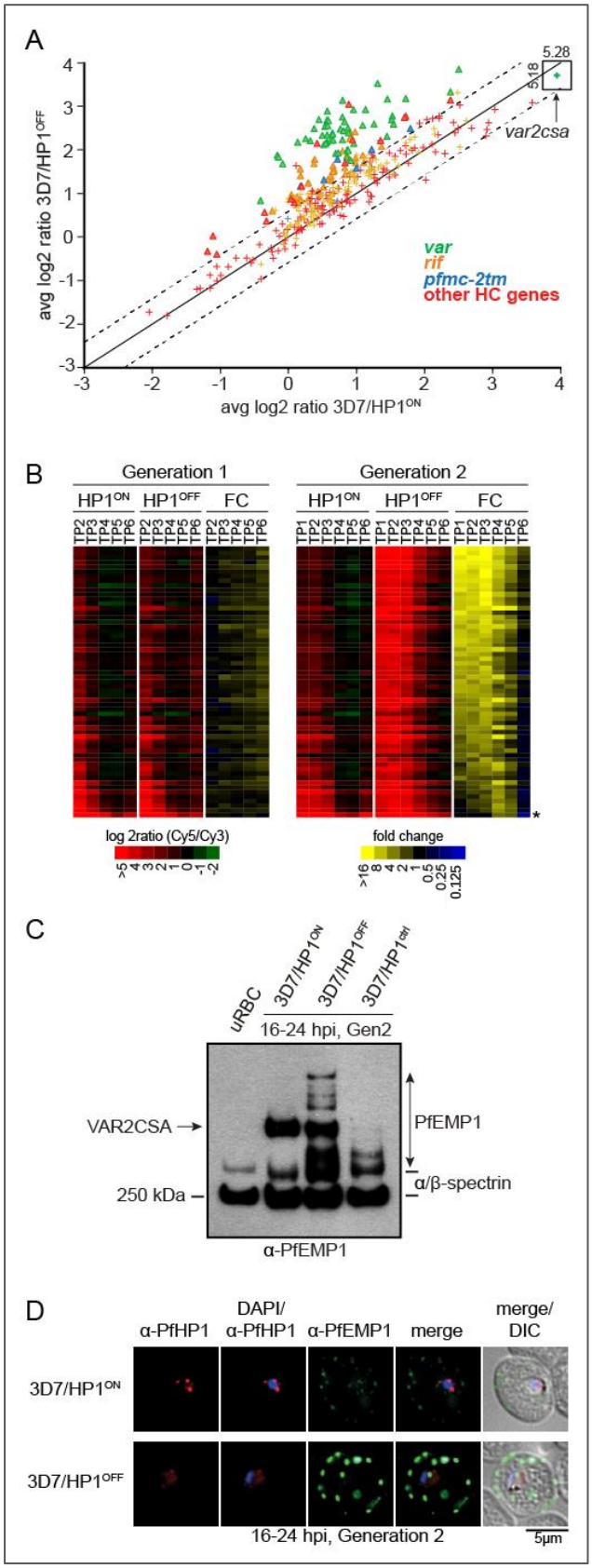


Figure 6. PfHP1 is required for heterochromatic gene silencing and maintenance of singular *var* gene choice.

(A) Scatter plot comparing the mean relative expression values (TPs 4-9) of 349 heterochromatic (HC) genes between 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites. Significantly dysregulated genes are indicated by triangles (>1.5-fold; fdr<0.1). (B) Temporal progression of relative abundance (red/green) and fold change (FC) in expression (yellow/blue) of all *var* genes in 3D7/HP1^{OFF} compared to 3D7/HP1^{ON} parasites. Asterisk, *var2csa* (PF3D7_1200600). (C) PfEMP1 expression in 3D7/HP1^{ON}, 3D7/HP1^{OFF} and 3D7/HP1^{ctrl} parasites (α-PfEMP1 antibodies cross-react with human spectrin). Equal cell numbers were analysed in each lane. uRBC, uninfected RBCs. (D) IFA images at 16-24 hpi in generation 2 using antibodies against PfHP1 and PfEMP1.

IFAs confirmed this PfEMP1 hyper-expression phenotype at the single cell level and further indicated correct trafficking of PfEMP1 to the iRBC surface (Figure 6D). Hence, PfHP1 depletion leads to the simultaneous transcription of most/all *var* genes and thus the disruption of singular gene choice.

4.4.6. Sexual Differentiation is Linked to the PfHP1-Dependent De-Repression of an ApiAP2 Transcription Factor

In contrast to the large number of heterochromatic loci, only 18 euchromatic genes were differentially expressed in 3D7/HP1^{OFF} parasites (12 up-regulated genes, 6 down-regulated genes) (>1.5-fold; fdr<0.1) (Figure 7A and Table S1). Three up-regulated genes are located adjacent to subtelomeric heterochromatin (PF3D7_0424400 (*surfin*), PF3D7_1301800 (*surfin* pseudogene), PF3D7_1252500), one gene encodes a putative steroid dehydrogenase (PF3D7_0422000), and two genes code for proteins with unknown function. Interestingly, 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), PF3D7_1473700 (*nup116*) (Silvestrini et al., 2010; Eksi et al., 2012). The most highly up-regulated genes, however, were two euchromatic PfHP1 target genes; *msp3.8* (PF3D7_1036300), which encodes a clonally variant surface antigen (Amambua-Ngwa et al., 2012); and PF3D7_1222600, a member of the *apiap2* family encoding lineage-specific TFs (Balaji et al., 2005). Remarkably, this *apiap2* gene is essential for gametocyte conversion in both *P. falciparum* and *P. berghei* (PBANKA_143750) and has thus been termed AP2-G (M. Llinás and A. Waters, personal communication).

We therefore investigated the likely causal relationship between PfHP1-dependent regulation of *ap2-g* and gametocyte conversion. As shown in Figure 7B, *ap2-g* de-repression was initiated in early schizonts and continued throughout further development, and was associated with the dissociation of PfHP1 from the *ap2-g* locus. Importantly, gametocyte hyper-conversion was prevented if 3D7/HP1^{OFF} parasites were allowed to re-accumulate PfHP1 prior to schizogony (28-36 hpi) (Figures 7B and S7). Restoring PfHP1 expression at as late as 34-42 hpi already caused a marked increase in sexual conversion, and parasites rescued at 40-48 hpi or after re-invasion showed a hyper-conversion phenotype similar to non-rescued parasites. These experiments map the critical time window for sexual commitment to mid/late schizogony (~36-44 hpi). Hence, the temporal correlation between de-repression of *ap2-g* and gametocyte commitment, together with the fact that both

processes are strictly dependent on PfHP1, identify the targeted activation of AP2-G as the only plausible mechanism responsible for sexual differentiation.

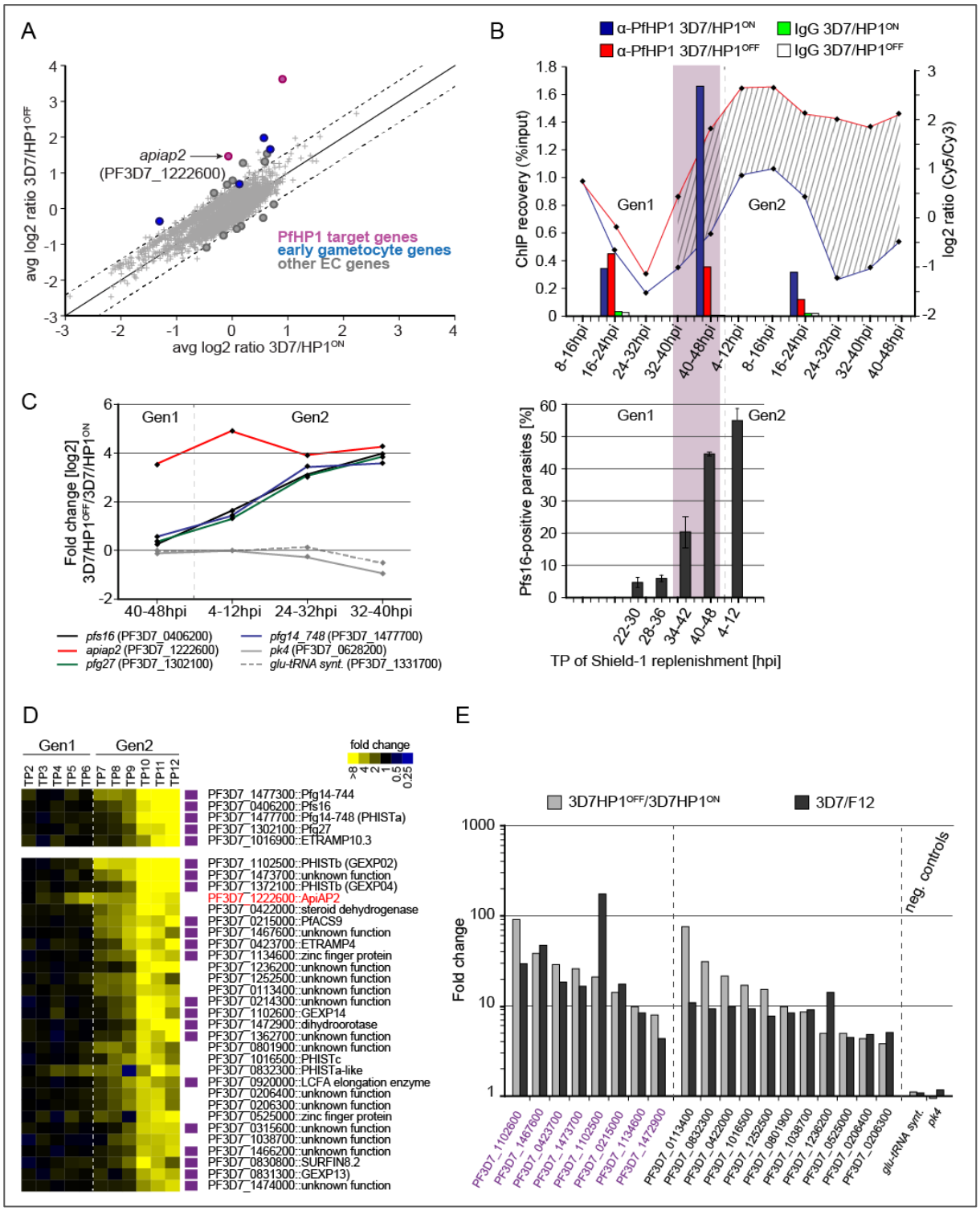


Figure 7. Gametocyte differentiation is linked to the PfHP1-dependent activation of *ap2-g* and up-regulation of early gametocyte genes.

(A) Scatter plot comparing the mean relative expression values (TPs 4-9) of 4784 euchromatic (EC) genes between 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites. Significantly de-regulated genes are indicated by circles (>1.5-fold; fdr<0.1). (B) Upper graph: PfHP1 occupancy at (left axis) and relative expression of (right axis) the *ap2-g* locus in 3D7/HP1^{ON} (blue) and 3D7/HP1^{OFF} (red) parasites (the shaded area marks >2-fold induction of

expression). Lower graph: Proportion of Pfs16-positive parasites in rescued 3D7/HP1^{OFF} populations as determined by IFA at 32-40 hours post re-invasion. Values show the mean of three biological replicates (+/- SD) (>100 parasites counted for each experiment). The commitment phase is highlighted in purple. (C) Temporal activation profile of *ap2-g* and three early gametocyte markers in 3D7/HP1^{OFF} parasites as determined by qRT-PCR on biological replicate samples. Negative control genes are in grey. (D) Temporal activation profile of known (upper heat map) and predicted (lower heat map) early gametocyte genes in 3D7/HP1^{OFF} parasites (microarray data). Previously identified early gametocyte genes are highlighted in purple. (E) Confirmation by qRT-PCR of induction of early gametocyte genes in 3D7/HP1^{OFF} compared to 3D7/HP1^{ON}, and in 3D7 wild type compared to gametocyte-deficient F12 parasites. Values represent the maximal fold change in relative expression out of four and three TPs analysed for the 3D7/HP1^{OFF}/3D7/HP1^{ON} and 3D7/F12 comparison (the full qRT-PCR datasets are presented in Figures S8 and S9).

4.4.7. 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 based on comparison of two isogenic clones. Consistent with the high rate at which PfHP1-depleted parasites differentiate into gametocytes, known markers of early sexual development were highly up-regulated in 3D7/HP1^{OFF} parasites (Figure 7C). Detailed examination of the transcriptome dataset identified 29 genes (excluding heterochromatic genes) with transcriptional profiles similar to those of five well-established early gametocyte markers, indicating a specific role for these genes during the initial phase of sexual differentiation (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. Validation by qRT-PCR on biological replicate samples confirmed up-regulation of these genes in 3D7/HP1^{OFF} parasites (Figures 7E and S8). Interestingly, apart from *ap2-g*, only one additional gene (PF3D7_0832300; *phista*-like) was up-regulated already during the commitment phase. Induction of other early gametocyte genes was delayed until the gametocyte ring stage and increased further during stage I development (24-40 hpi) (Figures 7C, 7D and S8). Finally, we tested if these candidate genes are also up-regulated in naturally induced gametocytes by assessing their transcription in 3D7 wild type parasites and a gametocyte-deficient clone of 3D7 (F12) (Alano et al., 1995). Indeed, all predicted genes had consistently higher transcription levels in 3D7 compared to F12, which confirms that their activation is related to sexual differentiation and gametocyte development (Figures 7E and S9).

4.5. Discussion

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 remodelling 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 to a unique perinuclear *var* expression site (VES), and 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 PfHP1 is essential for the heritable silencing of *var* genes and for perpetuating mutually exclusive transcriptional control. Depletion of PfHP1 during schizogony prevents re-establishment of heterochromatin on newly replicated chromosomes, which consequently leads to the simultaneous activation of all *var* genes and concomitant hyper-expression of PfEMP1 in daughter cells. A similar phenotype was recently reported after knockout of a H3K36-specific histone methyltransferase (PfSETvs) (Jiang et al., 2013). Interestingly, H3K36me3 as well as H3K9me3 occupancy was reduced at active *var* loci in these parasites suggesting functional interdependence between both silencing pathways.

Thus it appears that PfHP1 is required to protect *var* genes from activation outside the VES. This is 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, the functional principle of the VES in *P. falciparum* is not based on the sequestration of an exclusive transcription machinery but rather on histone modifying and remodelling activities capable of disassembling heterochromatin at a single locus. This concept is consistent with the recent description of a H3K4-specific HKMT (PfSET10) that localises exclusively to the VES (Volz et al., 2012). Taken together, these findings significantly advance our understanding of the molecular mechanisms underlying singular *var* gene choice and may help to explain conceptually similar processes in other organisms. For instance, it will be interesting to test if mammalian HP1 is also critical in regulating singular choice in odorant receptor (OR) gene expression,

where silenced and active states are also marked by H3K9me3 and H3K4me3, respectively (Magklara et al., 2011).

HP1 depletion also alleviated silencing of most other heterochromatic genes. However, the extent at which individual genes were de-repressed varied greatly. Several reasons could account for such differential responsiveness including limited spatial and/or temporal availability of TFs, local differences in the efficiency of PfHP1 depletion, proximity of genes to heterochromatin nucleation sites, or dependency on additional histone modifying and/or chromatin remodelling processes.

Apart from heterochromatic gene silencing, we discovered essential roles for PfHP1 in cell proliferation and differentiation. In absence of PfHP1, asexual trophozoites failed to proliferate and entered a state of cell cycle arrest that was reversible in a PfHP1-dependent manner. The exact pathway in which PfHP1 is required for cell cycle progression remains unknown. However, the lack of significant levels of DNA synthesis in PfHP1-depleted trophozoites is indicative of defects in S-phase entry or progression. Notably, 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; De et al., 2005). 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 detail.

More than 20 years ago, Bruce et al. showed that gametocyte commitment occurs during the asexual cell cycle preceding gametocyte formation and that all merozoites released from a committed schizont undergo sexual development (Bruce et al., 1990). Here, we demonstrate for the first time that the irreversible switch from vegetative growth to sexual development is epigenetically controlled by PfHP1. Depletion of PfHP1 in mid/late schizonts increased the proportion of parasites committing to sexual development by more than 20-fold. This unprecedented phenotype correlated with the targeted activation of *ap2-g*, which encodes an ApiAP2 TF essential for gametocyte conversion (M. Llinás and A. Waters, personal communication). Hence, our data provide compelling evidence for a first mechanistic model of sexual commitment in malaria parasites. We postulate that PfHP1-dependent silencing of *ap2-g* prevents gametocyte conversion and facilitates continuous mitotic proliferation cycles during blood stage infection. Local dissociation of PfHP1 from the *ap2-g* locus activates

AP2-G expression, which in turn triggers sexual conversion and gametocyte development. To our knowledge, this is the first example in unicellular eukaryotes where HP1 controls cell fate decision through the reversible silencing of a euchromatic gene. In this context it is interesting to note that while silencing of *var* and other heterochromatic genes is also dependent on PfSIR2A, PfSIR2B and PfSETvs (Tonkin et al., 2009; Duraisingh et al., 2005; Jiang et al., 2013), these histone modifiers are not involved in regulating *ap2-g* expression. PfHP1 therefore represents the only epigenetic regulator known so far to silence euchromatic genes in *P. falciparum*.

In analogy to the essential role of other 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), we predict AP2-G regulates a transcriptional response effecting gametocyte development and cell cycle exit. 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 merozoites capable of invading and remodelling RBCs. In fact, many of the early gametocyte genes predicted here and elsewhere code for proteins implicated in host cell remodelling (Eksi et al., 2005; Silvestrini et al., 2010). It is possible that AP2-G may regulate some of these genes directly, yet genome-wide ChIP approaches will be necessary for a comprehensive identification of AP2-G target genes and understanding AP2-G function.

Noteworthy, 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. Epigenetic control of this switch is therefore ideally suited to adapt gametocyte commitment rates during each mitotic proliferation cycle either stochastically or by responding rapidly to environmental triggers. Indeed, the rate at which gametocyte conversion occurs is highly variable between different isolates and clones and influenced by a broad spectrum of environmental conditions (Alano and Carter, 1990; Baker, 2010). Although the molecular factor(s) that trigger gametocyte conversion have not been identified, two seminal studies recently reported that cell-cell communication via iRBCs-derived exosomes or microvesicles leads to 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 signalling cascade that ultimately evicts PfHP1 from the *ap2-g* locus. In fact, phosphorylation of HP1 and the H3K9me3-adjacent serine residue on H3 have been reported as predominant mechanisms for the dissociation of HP1 from chromatin (Ayoub et al., 2008; Lomberk et al.,

2006a; Fischle et al., 2005). Since PfHP1 is also phosphorylated *in vivo* (Treeck et al., 2011) it is conceivable that similar mechanisms may be in place to dynamically regulate the association of PfHP1 with the *ap2-g* locus.

In conclusion, we identified fundamental roles for PfHP1 in proliferation, immune evasion and transmission of malaria blood stage parasites. Our data provide important mechanistic insight into the epigenetic processes underlying mutually exclusive *var* expression and antigenic variation of PfEMP1. Moreover, the PfEMP1 hyper-expression phenotype reported here and elsewhere (Jiang et al., 2013) may reveal opportunities for the development of anti-disease strategies targeting PfEMP1. Importantly, we also established for the first time that gametocyte commitment is regulated epigenetically by PfHP1. This significant discovery opens up unprecedented avenues for a targeted dissection of the molecular pathway triggering sexual conversion and will be highly relevant for the identification of novel approaches to prevent malaria transmission.

4.6. Experimental Procedures

Parasite Culture and Transfection

P. falciparum 3D7 cell culture and transfection were performed as described (Trager and Jenson, 1978; Lambros and Vanderberg, 1979; Voss et al., 2006). Transfection constructs are described in the Extended Experimental Procedures section. Parasites were grown in presence of indicated combinations of 4nM WR99210 (WR), 625nM Shield-1, 5µg/ml blasticidin-S-HCl. 3D7/HP1^{ON} clones were obtained by limiting dilution.

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 and α-H4 (Abcam ab10158), 1:10,000. PfEMP1 was extracted as described (van Schravendijk et al., 1993) and detected using mouse mAb 1B/6H-1 (1:500) (Duffy et al., 2002).

Fluorescence Microscopy

Live cell fluorescence microscopy was 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.

Isothermal Microcalorimetry

Isothermal microcalorimetry and data analysis were performed as originally described (Wenzler et al., 2012) with minor modifications (see Extended Experimental Procedures).

qRT-PCR and ChIP-qPCR

qRT-PCR and ChIP-qPCR was conducted as described with minor modifications (Witmer et al., 2012; Flueck et al., 2009). Detailed protocols are provided in the Extended Experimental Procedures section.

Flow Cytometry

Fixed parasites samples were analysed using an AccuriC6 instrument (BD Biosciences) after staining genomic DNA with FxCycle^{TM} Far Red stain (Molecular Probes). A detailed protocol for sample preparation and analysis is provided in the Extended Experimental Procedures section.

Microarray hybridisation and data analysis

Sample processing and microarray hybridisation was carried out as previously described (Bozdech et al., 2003; Hu et al., 2007). Detailed protocols describing the experimental workflow, microarray re-annotation, data processing and analysis are provided in the Extended Experimental Procedures section.

4.7. Acknowledgements

We thank M. Filarsky for critically reading the manuscript. We are grateful to R. Sauerwein and D. Taylor for providing antibodies, to M. Jud and M. Tamborrini for help in IgG subclass typing, and to C. Gysel and T. N. H. Bui for technical assistance. This work was

supported by the Swiss National Science Foundation (grant numbers PP00P3_130203, 31003A_143916, 310030_134889), the Singaporean National Medical Research Council (grant number NMRC/CBRG/0001/2012), the OPO Foundation and the Rudolf Geigy Foundation. N.M.B.B received a Boehringer Ingelheim PhD scholarship. The authors declare that no conflict of interest exists.

4.8. References

Aagaard,L., Laible,G., Selenko,P., Schmid,M., Dorn,R., Schotta,G., Kuhfittig,S., Wolf,A., Lebersorger,A., Singh,P.B., Reuter,G., and Jenuwein,T. (1999). Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31. EMBO J. 18, 1923-1938.

Alano,P. and Carter,R. (1990). Sexual differentiation in malaria parasites. Annu. Rev. Microbiol. 44, 429-449.

Alano,P., Roca,L., Smith,D., Read,D., Carter,R., and Day,K. (1995). Plasmodium falciparum: parasites defective in early stages of gametocytogenesis. Exp. Parasitol. 81, 227-235.

Amambua-Ngwa,A., Tetteh,K.K., Manske,M., Gomez-Escobar,N., Stewart,L.B., Deerhake,M.E., Cheeseman,I.H., Newbold,C.I., Holder,A.A., Knuepfer,E., Janha,O., Jallow,M., Campino,S., Macinnis,B., Kwiatkowski,D.P., and Conway,D.J. (2012). Population genomic scan for candidate signatures of balancing selection to guide antigen characterization in malaria parasites. PLoS. Genet. 8, e1002992.

Armstrong,C.M. and Goldberg,D.E. (2007). An FKBP destabilization domain modulates protein levels in Plasmodium falciparum. Nat. Methods 4, 1007-1009.

Ayoub,N., Jeyasekharan,A.D., Bernal,J.A., and Venkitaraman,A.R. (2008). HP1-beta mobilization promotes chromatin changes that initiate the DNA damage response. Nature 453, 682-686.

Baker,D.A. (2010). Malaria gametocytogenesis. Mol. Biochem. Parasitol. 172, 57-65.

Balaji,S., Babu,M.M., Iyer,L.M., and Aravind,L. (2005). Discovery of the principal specific transcription factors of Apicomplexa and their implication for the evolution of the AP2-integrase DNA binding domains. Nucleic Acids Res. 33, 3994-4006.

Banaszynski,L.A., Chen,L.C., Maynard-Smith,L.A., Ooi,A.G., and Wandless,T.J. (2006). A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. Cell 126, 995-1004.

Baruch,D.I., Pasloske,B.L., Singh,H.B., Bi,X., Ma,X.C., Feldman,M., Taraschi,T.F., and Howard,R.J. (1995). Cloning the P. falciparum gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. Cell 82, 77-87.

Baum,J., Papenfuss,A.T., Mair,G.R., Janse,C.J., Vlachou,D., Waters,A.P., Cowman,A.F., Crabb,B.S., and de Koning-Ward,T.F. (2009). Molecular genetics and comparative genomics reveal RNAi is not functional in malaria parasites. Nucleic Acids Res.

Bozdech,Z., Llinas,M., Pulliam,B.L., Wong,E.D., Zhu,J., and DeRisi,J.L. (2003). The transcriptome of the intraerythrocytic developmental cycle of Plasmodium falciparum. PLoS. Biol. 1, E5.

Brancucci,N.M., Witmer,K., Schmid,C.D., Flueck,C., and Voss,T.S. (2012). Identification of a cis-acting DNA-protein interaction implicated in singular var gene choice in Plasmodium falciparum. Cell Microbiol.

Bruce,M.C., Alano,P., Duthie,S., and Carter,R. (1990). Commitment of the malaria parasite Plasmodium falciparum to sexual and asexual development. Parasitology 100 Pt 2, 191-200.

Bruce,M.C., Carter,R.N., Nakamura,K., Aikawa,M., and Carter,R. (1994). Cellular location and temporal expression of the Plasmodium falciparum sexual stage antigen Pfs16. Mol. Biochem. Parasitol. 65, 11-22.

Chookajorn,T., Dzikowski,R., Frank,M., Li,F., Jiwani,A.Z., Hartl,D.L., and Deitsch,K.W. (2007). Epigenetic memory at malaria virulence genes. Proc. Natl. Acad. Sci. U. S. A 104, 899-902.

Christensen,T.W. and Tye,B.K. (2003). Drosophila MCM10 interacts with members of the prereplication complex and is required for proper chromosome condensation. Mol. Biol. Cell 14, 2206-2215.

Coleman,B.I., Ribacke,U., Manary,M., Bei,A.K., Winzeler,E.A., Wirth,D.F., and Duraisingh,M.T. (2012). Nuclear repositioning precedes promoter accessibility and is linked to the switching frequency of a Plasmodium falciparum invasion gene. Cell Host. Microbe 12, 739-750.

Cortes,A., Crowley,V.M., Vaquero,A., and Voss,T.S. (2012). A view on the role of epigenetics in the biology of malaria parasites. PLoS. Pathog. 8, e1002943.

Cowieson,N.P., Partridge,J.F., Allshire,R.C., and McLaughlin,P.J. (2000). Dimerisation of a chromo shadow domain and distinctions from the chromodomain as revealed by structural analysis. Curr. Biol. 10, 517-525.

Cowman,A.F. and Crabb,B.S. (2006). Invasion of red blood cells by malaria parasites. Cell 124, 755-766.

Crowley,V.M., Rovira-Graells,N., de Pouplana,L.R., and Cortes,A. (2011). Heterochromatin formation in bistable chromatin domains controls the epigenetic repression of clonally variant Plasmodium falciparum genes linked to erythrocyte invasion. Mol. Microbiol.

De,L.F., Ni,J.Q., Vaillant,C., and Sun,F.L. (2005). HP1 modulates the transcription of cell-cycle regulators in Drosophila melanogaster. Nucleic Acids Res. 33, 2852-2858.

Duffy,M.F., Brown,G.V., Basuki,W., Krejany,E.O., Noviyanti,R., Cowman,A.F., and Reeder,J.C. (2002). Transcription of multiple var genes by individual, trophozoite-stage Plasmodium falciparum cells expressing a chondroitin sulphate A binding phenotype. Mol. Microbiol. 43, 1285-1293.

Duraisingh,M.T., Voss,T.S., Marty,A.J., Duffy,M.F., Good,R.T., Thompson,J.K., Freitas-Junior,L.H., Scherf,A., Crabb,B.S., and Cowman,A.F. (2005). Heterochromatin silencing and locus repositioning linked to regulation of virulence genes in Plasmodium falciparum. Cell 121, 13-24.

Eissenberg,J.C., James,T.C., Foster-Hartnett,D.M., Hartnett,T., Ngan,V., and Elgin,S.C. (1990). Mutation in a heterochromatin-specific chromosomal protein is associated with

suppression of position-effect variegation in Drosophila melanogaster. Proc. Natl. Acad. Sci. U. S. A 87, 9923-9927.

Eksi,S., Haile,Y., Furuya,T., Ma,L., Su,X., and Williamson,K.C. (2005). Identification of a subtelomeric gene family expressed during the asexual-sexual stage transition in Plasmodium falciparum. Mol. Biochem. Parasitol. 143, 90-99.

Eksi,S., Morahan,B.J., Haile,Y., Furuya,T., Jiang,H., Ali,O., Xu,H., Kiattibutr,K., Suri,A., Czesny,B., Adeyemo,A., Myers,T.G., Sattabongkot,J., Su,X.Z., and Williamson,K.C. (2012). Plasmodium falciparum gametocyte development 1 (Pfgdv1) and gametocytogenesis early gene identification and commitment to sexual development. PLoS. Pathog. 8, e1002964.

Fernandez,V., Hommel,M., Chen,Q., Hagblom,P., and Wahlgren,M. (1999). Small, clonally variant antigens expressed on the surface of the Plasmodium falciparum-infected erythrocyte are encoded by the rif gene family and are the target of human immune responses. J. Exp. Med. 190, 1393-1404.

Fischle,W., Tseng,B.S., Dormann,H.L., Ueberheide,B.M., Garcia,B.A., Shabanowitz,J., Hunt,D.F., Funabiki,H., and Allis,C.D. (2005). Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. Nature 438, 1116-1122.

Flueck,C., Bartfai,R., Volz,J., Niederwieser,I., Salcedo-Amaya,A.M., Alako,B.T., Ehlgen,F., Ralph,S.A., Cowman,A.F., Bozdech,Z., Stunnenberg,H.G., and Voss,T.S. (2009). Plasmodium falciparum heterochromatin protein 1 marks genomic loci linked to phenotypic variation of exported virulence factors. PLoS Pathog. 5, e1000569.

Foth,B.J., Zhang,N., Chaal,B.K., Sze,S.K., Preiser,P.R., and Bozdech,Z. (2011). Quantitative time-course profiling of parasite and host cell proteins in the human malaria parasite Plasmodium falciparum. Mol. Cell Proteomics. 10, M110.

Freitas-Junior,L.H., Hernandez-Rivas,R., Ralph,S.A., Montiel-Condado,D., Ruvalcaba-Salazar,O.K., Rojas-Meza,A.P., Mancio-Silva,L., Leal-Silvestre,R.J., Gontijo,A.M., Shorte,S., and Scherf,A. (2005). Telomeric heterochromatin propagation and histone acetylation control mutually exclusive expression of antigenic variation genes in malaria parasites. Cell 121, 25-36.

Hall,I.M., Shankaranarayana,G.D., Noma,K., Ayoub,N., Cohen,A., and Grewal,S.I. (2002). Establishment and maintenance of a heterochromatin domain. Science 297, 2232-2237.

Hayashi,M.T., Takahashi,T.S., Nakagawa,T., Nakayama,J., and Masukata,H. (2009). The heterochromatin protein Swi6/HP1 activates replication origins at the pericentromeric region and silent mating-type locus. Nat. Cell Biol. 11, 357-362.

Hediger,F. and Gasser,S.M. (2006). Heterochromatin protein 1: don't judge the book by its cover! Curr. Opin. Genet. Dev. 16, 143-150.

Hu,G., Llinas,M., Li,J., Preiser,P.R., and Bozdech,Z. (2007). Selection of long oligonucleotides for gene expression microarrays using weighted rank-sum strategy. BMC. Bioinformatics. 8, 350.

Iwanaga,S., Kaneko,I., Kato,T., and Yuda,M. (2012). Identification of an AP2-family protein that is critical for malaria liver stage development. PLoS. ONE. 7, e47557.

Jiang,L., Lopez-Barragan,M.J., Jiang,H., Mu,J., Gaur,D., Zhao,K., Felsenfeld,G., and Miller,L.H. (2010). Epigenetic control of the variable expression of a Plasmodium

falciparum receptor protein for erythrocyte invasion. Proc. Natl. Acad. Sci. U. S. A 107, 2224-2229.

Jiang,L., Mu,J., Zhang,Q., Ni,T., Srinivasan,P., Rayavara,K., Yang,W., Turner,L., Lavstsen,T., Theander,T.G., Peng,W., Wei,G., Jing,Q., Wakabayashi,Y., Bansal,A., Luo,Y., Ribeiro,J.M., Scherf,A., Aravind,L., Zhu,J., Zhao,K., and Miller,L.H. (2013). PfSETvs methylation of histone H3K36 represses virulence genes in Plasmodium falciparum. Nature 499, 223-227.

Kanellopoulou,C., Muljo,S.A., Kung,A.L., Ganesan,S., Drapkin,R., Jenuwein,T., Livingston,D.M., and Rajewsky,K. (2005). Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. Genes Dev. 19, 489-501.

Kwon,S.H. and Workman,J.L. (2008). The heterochromatin protein 1 (HP1) family: put away a bias toward HP1. Mol. Cells 26, 217-227.

Kyes,S., Horrocks,P., and Newbold,C. (2001). Antigenic variation at the infected red cell surface in malaria. Annu. Rev. Microbiol. 55, 673-707.

Lachner,M., O'Carroll,D., Rea,S., Mechtler,K., and Jenuwein,T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature 410, 116-120.

Lambros,C. and Vanderberg,J.P. (1979). Synchronization of Plasmodium falciparum erythrocytic stages in culture. J. Parasitol. 65, 418-420.

Lavazec,C., Sanyal,S., and Templeton,T.J. (2007). Expression switching in the stevor and Pfmc-2TM superfamilies in Plasmodium falciparum. Mol. Microbiol. 64, 1621-1634.

Leech,J.H., Barnwell,J.W., Miller,L.H., and Howard,R.J. (1984). Identification of a strain-specific malarial antigen exposed on the surface of Plasmodium falciparum-infected erythrocytes. J. Exp. Med. 159, 1567-1575.

Li,P.C., Chretien,L., Cote,J., Kelly,T.J., and Forsburg,S.L. (2011). S. pombe replication protein Cdc18 (Cdc6) interacts with Swi6 (HP1) heterochromatin protein: region specific effects and replication timing in the centromere. Cell Cycle 10, 323-336.

Lomberk,G., Bensi,D., Fernandez-Zapico,M.E., and Urrutia,R. (2006a). Evidence for the existence of an HP1-mediated subcode within the histone code. Nat. Cell Biol. 8, 407-415.

Lomberk,G., Wallrath,L., and Urrutia,R. (2006b). The Heterochromatin Protein 1 family. Genome Biol. 7, 228.

Lopez-Rubio,J.J., Gontijo,A.M., Nunes,M.C., Issar,N., Hernandez,R.R., and Scherf,A. (2007). 5' flanking region of var genes nucleate histone modification patterns linked to phenotypic inheritance of virulence traits in malaria parasites. Mol. Microbiol. 66, 1296-1305.

Lopez-Rubio,J.J., Mancio-Silva,L., and Scherf,A. (2009). Genome-wide analysis of heterochromatin associates clonally variant gene regulation with perinuclear repressive centers in malaria parasites. Cell Host. Microbe 5, 179-190.

Magklara,A., Yen,A., Colquitt,B.M., Clowney,E.J., Allen,W., Markenscoff-Papadimitriou,E., Evans,Z.A., Kheradpour,P., Mountoufaris,G., Carey,C., Barnea,G., Kellis,M., and Lomvardas,S. (2011). An epigenetic signature for monoallelic olfactory receptor expression. Cell 145, 555-570.

Mantel,P.Y., Hoang,A.N., Goldowitz,I., Potashnikova,D., Hamza,B., Vorobjev,I., Ghiran,I., Toner,M., Irimia,D., Ivanov,A.R., Barteneva,N., and Marti,M. (2013). Malaria-infected

erythrocyte-derived microvesicles mediate cellular communication within the parasite population and with the host immune system. Cell Host. Microbe 13, 521-534.

Mok,S., Imwong,M., Mackinnon,M.J., Sim,J., Ramadoss,R., Yi,P., Mayxay,M., Chotivanich,K., Liong,K.Y., Russell,B., Socheat,D., Newton,P.N., Day,N.P., White,N.J., Preiser,P.R., Nosten,F., Dondorp,A.M., and Bozdech,Z. (2011). Artemisinin resistance in Plasmodium falciparum is associated with an altered temporal pattern of transcription. BMC. Genomics 12, 391.

Nguitragool,W., Bokhari,A.A., Pillai,A.D., Rayavara,K., Sharma,P., Turpin,B., Aravind,L., and Desai,S.A. (2011). Malaria parasite clag3 genes determine channel-mediated nutrient uptake by infected red blood cells. Cell 145, 665-677.

Pal-Bhadra,M., Leibovitch,B.A., Gandhi,S.G., Rao,M., Bhadra,U., Birchler,J.A., and Elgin,S.C. (2004). Heterochromatic silencing and HP1 localization in Drosophila are dependent on the RNAi machinery. Science 303, 669-672.

Perez-Toledo,K., Rojas-Meza,A.P., Mancio-Silva,L., Hernandez-Cuevas,N.A., Delgadillo,D.M., Vargas,M., Martinez-Calvillo,S., Scherf,A., and Hernandez-Rivas,R. (2009). Plasmodium falciparum heterochromatin protein 1 binds to tri-methylated histone 3 lysine 9 and is linked to mutually exclusive expression of var genes. Nucleic Acids Res. 37, 2596-2606.

Petter,M., Haeggstrom,M., Khattab,A., Fernandez,V., Klinkert,M.Q., and Wahlgren,M. (2007). Variant proteins of the Plasmodium falciparum RIFIN family show distinct subcellular localization and developmental expression patterns. Mol. Biochem. Parasitol. 156, 51-61.

Petter,M., Lee,C.C., Byrne,T.J., Boysen,K.E., Volz,J., Ralph,S.A., Cowman,A.F., Brown,G.V., and Duffy,M.F. (2011). Expression of P. falciparum var genes involves exchange of the histone variant H2A.Z at the promoter. PLoS Pathog. 7, e1001292.

Quivy,J.P., Gerard,A., Cook,A.J., Roche,D., and Almouzni,G. (2008). The HP1-p150/CAF-1 interaction is required for pericentric heterochromatin replication and S-phase progression in mouse cells. Nat. Struct. Mol. Biol. 15, 972-979.

Ralph,S.A., Scheidig-Benatar,C., and Scherf,A. (2005). Antigenic variation in Plasmodium falciparum is associated with movement of var loci between subnuclear locations. Proc. Natl. Acad. Sci. U. S. A 102, 5414-5419.

Regev-Rudzki,N., Wilson,D.W., Carvalho,T.G., Sisquella,X., Coleman,B.M., Rug,M., Bursac,D., Angrisano,F., Gee,M., Hill,A.F., Baum,J., and Cowman,A.F. (2013). Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. Cell 153, 1120-1133.

Reuter,G. and Spierer,P. (1992). Position effect variegation and chromatin proteins. Bioessays 14, 605-612.

Rovira-Graells,N., Gupta,A.P., Planet,E., Crowley,V.M., Mok,S., Ribas de,P.L., Preiser,P.R., Bozdech,Z., and Cortes,A. (2012). Transcriptional variation in the malaria parasite Plasmodium falciparum. Genome Res 22, 925-938.

Salanti,A., Staalsoe,T., Lavstsen,T., Jensen,A.T., Sowa,M.P., Arnot,D.E., Hviid,L., and Theander,T.G. (2003). Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria. Mol. Microbiol. 49, 179-191.

Salcedo-Amaya,A.M., van Driel,M.A., Alako,B.T., Trelle,M.B., van den Elzen,A.M., Cohen,A.M., Janssen-Megens,E.M., van,d., V, Selzer,R.R., Iniguez,A.L., Green,R.D., Sauerwein,R.W., Jensen,O.N., and Stunnenberg,H.G. (2009). Dynamic histone H3 epigenome marking during the intraerythrocytic cycle of Plasmodium falciparum. Proc. Natl. Acad. Sci. U. S. A 106, 9655-9660.

Sargeant,T.J., Marti,M., Caler,E., Carlton,J.M., Simpson,K., Speed,T.P., and Cowman,A.F. (2006). Lineage-specific expansion of proteins exported to erythrocytes in malaria parasites. Genome Biol. 7, R12.

Scherf,A., Hernandez-Rivas,R., Buffet,P., Bottius,E., Benatar,C., Pouvelle,B., Gysin,J., and Lanzer,M. (1998). Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum. EMBO J. 17, 5418-5426.

Schwaiger,M., Kohler,H., Oakeley,E.J., Stadler,M.B., and Schubeler,D. (2010). Heterochromatin protein 1 (HP1) modulates replication timing of the Drosophila genome. Genome Res. 20, 771-780.

Silvestrini,F., Bozdech,Z., Lanfrancotti,A., Di,G.E., Bultrini,E., Picci,L., DeRisi,J.L., Pizzi,E., and Alano,P. (2005). Genome-wide identification of genes upregulated at the onset of gametocytogenesis in Plasmodium falciparum. Mol. Biochem. Parasitol. 143, 100-110.

Silvestrini,F., Lasonder,E., Olivieri,A., Camarda,G., van,S.B., Sanchez,M., Younis,Y.S., Sauerwein,R., and Alano,P. (2010). Protein export marks the early phase of gametocytogenesis of the human malaria parasite plasmodium falciparum. Mol. Cell Proteomics. 9, 1437-1448.

Smith,J.D., Chitnis,C.E., Craig,A.G., Roberts,D.J., Hudson-Taylor,D.E., Peterson,D.S., Pinches,R., Newbold,C.I., and Miller,L.H. (1995). Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. Cell 82, 101-110.

Su,X.Z., Heatwole,V.M., Wertheimer,S.P., Guinet,F., Herrfeldt,J.A., Peterson,D.S., Ravetch,J.A., and Wellems,T.E. (1995). The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. Cell 82, 89-100.

Taylor,D.W., Parra,M., Chapman,G.B., Stearns,M.E., Rener,J., Aikawa,M., Uni,S., Aley,S.B., Panton,L.J., and Howard,R.J. (1987). Localization of Plasmodium falciparum histidine-rich protein 1 in the erythrocyte skeleton under knobs. Mol. Biochem. Parasitol. 25, 165-174.

Tonkin,C.J., Carret,C.K., Duraisingh,M.T., Voss,T.S., Ralph,S.A., Hommel,M., Duffy,M.F., Silva,L.M., Scherf,A., Ivens,A., Speed,T.P., Beeson,J.G., and Cowman,A.F. (2009). Sir2 paralogues cooperate to regulate virulence genes and antigenic variation in Plasmodium falciparum. PLoS. Biol. 7, e84.

Trager,W. and Jenson,J.B. (1978). Cultivation of malarial parasites. Nature 273, 621-622.

Treeck,M., Sanders,J.L., Elias,J.E., and Boothroyd,J.C. (2011). The phosphoproteomes of Plasmodium falciparum and Toxoplasma gondii reveal unusual adaptations within and beyond the parasites' boundaries. Cell Host. Microbe 10, 410-419.

van Schravendijk,M.R., Pasloske,B.L., Baruch,D.I., Handunnetti,S.M., and Howard,R.J. (1993). Immunochemical characterization and differentiation of two approximately 300-

kD erythrocyte membrane-associated proteins of Plasmodium falciparum, PfEMP1 and PfEMP3. Am. J. Trop. Med. Hyg. 49, 552-565.

Volz,J.C., Bartfai,R., Petter,M., Langer,C., Josling,G.A., Tsuboi,T., Schwach,F., Baum,J., Rayner,J.C., Stunnenberg,H.G., Duffy,M.F., and Cowman,A.F. (2012). PfSET10, a Plasmodium falciparum methyltransferase, maintains the active var gene in a poised state during parasite division. Cell Host. Microbe 11, 7-18.

Voss,T.S., Healer,J., Marty,A.J., Duffy,M.F., Thompson,J.K., Beeson,J.G., Reeder,J.C., Crabb,B.S., and Cowman,A.F. (2006). A var gene promoter controls allelic exclusion of virulence genes in Plasmodium falciparum malaria. Nature 439, 1004-1008.

Voss,T.S., Kaestli,M., Vogel,D., Bopp,S., and Beck,H.P. (2003). Identification of nuclear proteins that interact differentially with Plasmodium falciparum var gene promoters. Mol. Microbiol. 48, 1593-1607.

Wenzler,T., Steinhuber,A., Wittlin,S., Scheurer,C., Brun,R., and Trampuz,A. (2012). Isothermal microcalorimetry, a new tool to monitor drug action against Trypanosoma brucei and Plasmodium falciparum. PLoS. Negl. Trop. Dis. 6, e1668.

Witmer,K., Schmid,C.D., Brancucci,N.M., Luah,Y.H., Preiser,P.R., Bozdech,Z., and Voss,T.S. (2012). Analysis of subtelomeric virulence gene families in Plasmodium falciparum by comparative transcriptional profiling. Mol. Microbiol. 84, 243-259.

World Health Organisation (2012). World Malaria Report 2012. (Geneva: WHO Press).

Yamamoto,K. and Sonoda,M. (2003). Self-interaction of heterochromatin protein 1 is required for direct binding to histone methyltransferase, SUV39H1. Biochem. Biophys. Res. Commun. 301, 287-292.

Young,J.A., Fivelman,Q.L., Blair,P.L., de,l., V, Le Roch,K.G., Zhou,Y., Carucci,D.J., Baker,D.A., and Winzeler,E.A. (2005). The Plasmodium falciparum sexual development transcriptome: a microarray analysis using ontology-based pattern identification. Mol. Biochem. Parasitol. 143, 67-79.

Yuda,M., Iwanaga,S., Shigenobu,S., Kato,T., and Kaneko,I. (2010). Transcription factor AP2-Sp and its target genes in malarial sporozoites. Mol. Microbiol. 75, 854-863.

Yuda,M., Iwanaga,S., Shigenobu,S., Mair,G.R., Janse,C.J., Waters,A.P., Kato,T., and Kaneko,I. (2009). Identification of a transcription factor in the mosquito-invasive stage of malaria parasites. Mol. Microbiol. 71, 1402-1414.

4.9. Supporting Information

4.9.1. Extended Experimental Procedures

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 destabilising 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 645 bp fragment corresponding to the 3' end of *pfhp1* into *Pst*I/*Not*I-digested pH-GFP or pH-GFP-DD, respectively. pBcamHP1-Cherry was generated by inserting the PCR-amplified *pfhp1* full length coding sequence into *BamH*I/*Nhe*I-digested pBcam-3xHA-CherryFP (Witmer et al., 2012). All primer sequences are listed in Table S2.

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 immunization 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 neutralized 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 separately 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 immobilized on a Ni^{2+}-charged HiTrap IMAC FF column (GE Healthcare).

6ml of purified 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 stabilized 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 S2).

Quantitative Reverse Transcription PCR (qRT-PCR)

3D7/HP1^{ON} parasites were synchronized twice 16 hours apart to obtain an eight hour growth window and then split into two populations at 4-12 hpi and cultured either in presence or absence of Shield-1. 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites were harvested at 40-48 hpi in generation 1 and at three consecutive time points in generation 2 (4-12hpi, 24-32hpi, 32-40hpi). 3D7 and F12 populations were synchronized identically and time points were harvested at 4-12 hpi, 24-32 hpi and 40-48 hpi. 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*^{TM} 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^{TM} Real-Time PCR System (Applied Biosystems) in a reaction volume of 12ul. 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 normalization against the house-keeping gene arginyl-tRNA synthetase (PF3D7_1218600) (Frank et al., 2006). Primer sequences are listed in Table S2.

Targeted Chromatin Immunoprecipitation (ChIP-qPCR)

3D7/HP1^{ON} parasites were synchronized 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 4-12 hpi. Sample pairs were harvested at 16-24 hpi and 40-48 hpi in generation 1, and at 16-24 hpi 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 antibodies in presence of 20µl protein A/G agarose beads (Pierce) overnight at 4°C. Negative control ChIPs were performed using 1µg rabbit IgG (Upstate 12–370). Beads were washed six times and immunoprecipitated chromatin was eluted with 1% SDS, 0.1 M $NaHCO_3$. 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.

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 optimizations had to be applied. The sample volume was increased to 3 ml to enhance heat flow signal when using low starting parasitaemias. In addition, adjusting the parasitaemia of synchronized 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 synchronized parasite cultures is characterized 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 4-12 hpi 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 6-14 hpi (0.25% parasitaemia) and 28-36 hpi (0.15% parasitaemia) for measurements in generation 1 and 2, respectively.

Indirect Immunofluorescence Assays

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.

Flow Cytometry

Tightly synchronized 3D7/HP1^{ON} parasites were split at 0-4 hpi and cultured either in presence or absence of Shield-1. At 20-24 hpi after re-invasion the 3D7/HP1^{ON} and 3D7/HP1^{OFF} populations were synchronized again to obtain a tight four hour growth window. DNA content analysis was carried out on five consecutive time points in generation 2, starting at 24-28 hpi. 100µl packed RBCs were fixed in 4% formaldehyde/0.01% 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 30 min 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).

Microarray Experiments and Data Analysis

RNA extraction, labeling and microarray hybridization

Total RNA extraction and cDNA synthesis was carried out as described (Bozdech et al., 2003b). Cy5-labeled test cDNAs were hybridized against a Cy3-labeled cDNA reference pool that was made by combining total RNA isolated from 3D7 wild-type parasites harvested at five consecutive time points across the IDC. Equal amounts of Cy5- and Cy3-labeled samples were hybridized on a *P. falciparum* glass slide microarray containing 10416 70-mer ORF probes (Hu et al., 2007). Hybridization was carried out at 65°C in an automated hybridization station (Maui, USA) for at least 12 hours as described (Bozdech et al., 2003a). 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).

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 normalization 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 (Ritchie et al., 2007) and normalized by locally estimated scatterplot smoothing (LOESS) within arrays and quantile-normalized between arrays (Yang and Thorne, 2003). 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 final microarray dataset is 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 -π 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 (see table below).

	TP2	TP3	TP4	TP5	TP6	TP7	TP8	TP9	TP10	TP11	TP12
3D7/HP1^{ON}	0.79	0.89	0.85	0.80	0.82	0.75	0.77	0.88	0.83	0.79	0.78
3D7/HP1^{OFF}	0.80	0.88	0.84	0.75	0.78	0.65	0.69	0.75	0.78	0.73	0.39

Table: Values are Spearman rank correlation coefficients (ρ) between each experimental transcriptome and the best-fit reference time point in a high resolution transcriptome dataset (related to Figure 5B).

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.

4.9.2. Supporting Figure and Table Legends

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

(A) Schematic map of the endogenous *pfhp*1 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 lengths of the fragments are indicated and colour-coded. The labelled hybridization 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*-thymidylate synthase gene terminator; *gfp*, green fluorescent protein; *dd*, destabilization 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 hybridized with a ^{32}P-dATP-labeled *pfhp1* fragment.

Figure S2. Specificity of the polyclonal affinity-purified anti-*Pf*HP1 antibody (related to Extended Experimental Procedures)

α-PfHP1 Western blot analysis 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).

Figure S3. Complementation with PfHP1-CherryFP compensates for PfHP1-GFP-DD depletion (related to Figure 2)

(A) Schematic map of the complementation vector pBcamHP1-CherryFP. hsp86 5', heat shock protein 86 gene promoter; *bsd*, blasticidin deaminase; Pb DT 3', *P. berghei dhfr*-thymidilate synthase gene terminator; TARE 6, 0.5kb rep20 repeat sequence; cam 5', calmodulin gene promoter; Cherry, Cherry fluorescent protein; hrp2 3', histidine-rich protein 2 gene terminator. (B) Live fluorescence microscopy of 3D7/HP1^{ON} parasites expressing episomal PfHP1-Cherry (eHP1Cherry) reveals co-localization of PfHP1-GFP-DD and PfHP1-CherryFP at telomeric foci. PfHP1-GFP-DD, but not PfHP1-CherryFP, is efficiently depleted 12 hrs after Shield-1 removal. After withdrawal of Shield-1, parasites complemented with HP1-Cherry were able to progress through generation 2 and any following IDC. (C) Comparison of multiplication rates of complemented 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites over three generations.

Figure S4. Time required for restoring asexual growth after Shield-1 replenishment (related to Figure 4)

Synchronous 3D7/HP1^{ON} parasites at ~0.1% parasitaemia were split at 0-8 hpi and cultured either in presence or absence of Shield-1. Shield-1 was added back to 3D7/HP1^{OFF} cultures at nine consecutive time points. Cultures were smeared daily and analysed by Giemsa staining until they reached a parasitaemia of >1%. Values show the mean of three biological replicates (+/- SEM).

Figure S5. PfHP1-depletion causes de-repression of heterochromatic genes (related to Figure 6A)

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.

Figure S6. Absolute *var* transcript abundance in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites (related to Figure 6B)

Values represent the mean raw signal intensities for all *var* genes for TPs7-9 in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites (Cy5), and for all 3D7 reference pool hybridizations (Cy3) (+/- SEM). 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 S7. 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 4-12 hpi by Shield-1 removal (3D7/HP1^{OFF}) and subsequently restored (3D7/HP1$^{OFF\rightarrow ON}$) by replenishing Shield-1 at 16-24 hpi and 28-36 hpi. Images were taken at the indicated time points with identical settings.

Figure S8. Confirmation of induction of novel early gametocyte genes by qRT-PCR on biological replicate samples (related to Figure 7E)

qRT-PCR on biological replicate samples confirms the induction of commitment and early gametocyte genes in PfHP1-depleted parasites. PfHP1 depletion was induced 4-12 hpi 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, 40-48 hpi generation 1); gametocyte ring stage (TP2, 4-12 hpi generation 2); stage I gametocytes (TPs3 and 4, 24-32 hpi and 32-40 hpi). Relative expression values were normalized for arginyl tRNA-synthetase (PF3D7_1218600) transcripts.

Figure S9. qRT-PCR-based confirmation of induction of novel early gametocyte genes in 3D7 wild type parasites compared to the gametocyte-deficient clone F12 (related to Figure 7E)

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, 4-12 hpi); early schizonts (TP2, 32-40 hpi); late schizonts (TP3, 40-48 hpi). Relative expression values were normalized for arginyl tRNA-synthetase (PF3D7_1218600) transcripts.

Table S1: Final microarray dataset (related to Figures 5, 6, 7, S5 and S6)

Columns A-C: Basic gene information. Columns D-Y: averaged Cy5/Cy3 log2 ratios for all transcripts in every time point (TPs2-12) harvested from 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites. Columns Z-AA: Phase calculation values (see Extended Experimental Procedures). Columns AB-AD: Classification of genes into heterochromatic, euchromatic PfHP1-associated, and euchromatic groups based on the data published by Flueck et al. (Flueck et al., 2009) and manual curation. Columns AE-AJ: mean fold change in gene expression in 3D7/HP1^{OFF} compared to 3D7/HP1^{ON} parasites and SAM significance value (% FDR). Columns AM-AR: genes expressed in gametocytes based on published work. Column S: early gametocyte genes predicted in this study (see also Extended Experimental Procedures). Table S1 (Excel file) is given on the supporting CD-ROM.

Table S2: List of all primers used in this study (related to Extended 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	tggtggattggcacttataccttt	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**	

4.9.3. Supplemental References

Alano,P., Premawansa,S., Bruce,M.C., and Carter,R. (1991). A stage specific gene expressed at the onset of gametocytogenesis in Plasmodium falciparum. Mol. Biochem. Parasitol. *46*, 81-88.

Bozdech,Z., Zhu,J., Joachimiak,M.P., Cohen,F.E., Pulliam,B., and DeRisi,J.L. (2003b). Expression profiling of the schizont and trophozoite stages of Plasmodium falciparum with a long-oligonucleotide microarray. Genome Biol. *4*, R9.

Cinquin,O., Christopherson,R.I., and Menz,R.I. (2001). A hybrid plasmid for expression of toxic malarial proteins in Escherichia coli. Mol. Biochem. Parasitol. *117*, 245-247.

Dvorin,J.D., Martyn,D.C., Patel,S.D., Grimley,J.S., Collins,C.R., Hopp,C.S., Bright,A.T., Westenberger,S., Winzeler,E., Blackman,M.J., et al. (2010). A plant-like kinase in Plasmodium falciparum regulates parasite egress from erythrocytes. Science *328*, 910-912.

Eisen,M.B., Spellman,P.T., Brown,P.O., and Botstein,D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U. S. A *95*, 14863-14868.

Frank,M., Dzikowski,R., Costantini,D., Amulic,B., Berdougo,E., and Deitsch,K. (2006). Strict pairing of var promoters and introns is required for var gene silencing in the malaria parasite Plasmodium falciparum. J. Biol. Chem. *281*, 9942-9952.

Gu,J., Stephenson,C.G., and Iadarola,M.J. (1994). Recombinant proteins attached to a nickel-NTA column: use in affinity purification of antibodies. Biotechniques *17*, 257, 260, 262.

Ritchie,M.E., Silver,J., Oshlack,A., Holmes,M., Diyagama,D., Holloway,A., and Smyth,G.K. (2007). A comparison of background correction methods for two-colour microarrays. Bioinformatics. *23*, 2700-2707.

Saldanha,A.J. (2004). Java Treeview--extensible visualization of microarray data. Bioinformatics. *20*, 3246-3248.

Smyth,W.A. (2005). Limma: linear models for microarray data. In Bioinformatics and Computational Biology Solutions using R and Bioconductor, R.Gentleman, V.Carey, S.Dudoit, R.Irizarry, and W.Huber, eds. (New York: Springer), pp. 397-420.

Studier,F.W. (2005). Protein production by auto-induction in high density shaking cultures. Protein Expr. Purif. *41*, 207-234.

Tusher,V.G., Tibshirani,R., and Chu,G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U. S. A *98*, 5116-5121.

Yang, Y. H. and Thorne, N. P. (2003). Normalization for two-color cDNA microarray data. In Statistics and Science: A Festschrift for Terry Speed. D.R.Goldstein, ed. (Ohio, Institute of Mathematical Statistics), IMS Lecture Notes - Monograph Series, *40*, pp. 403-418.

4.9.4. Supplementary Figures

Figure S1

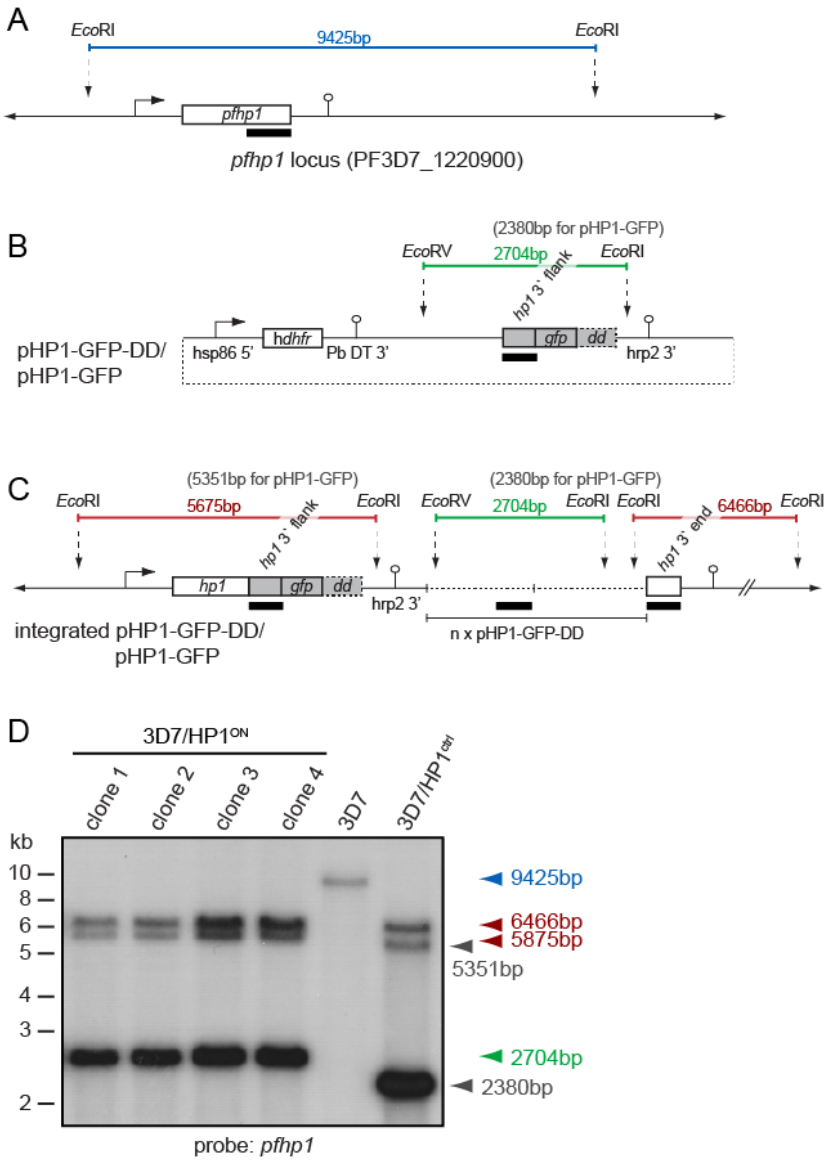


Figure S2

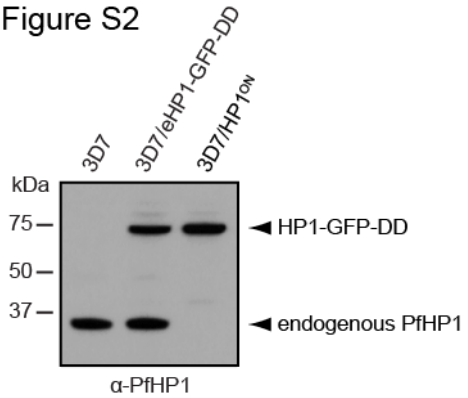


Figure S3

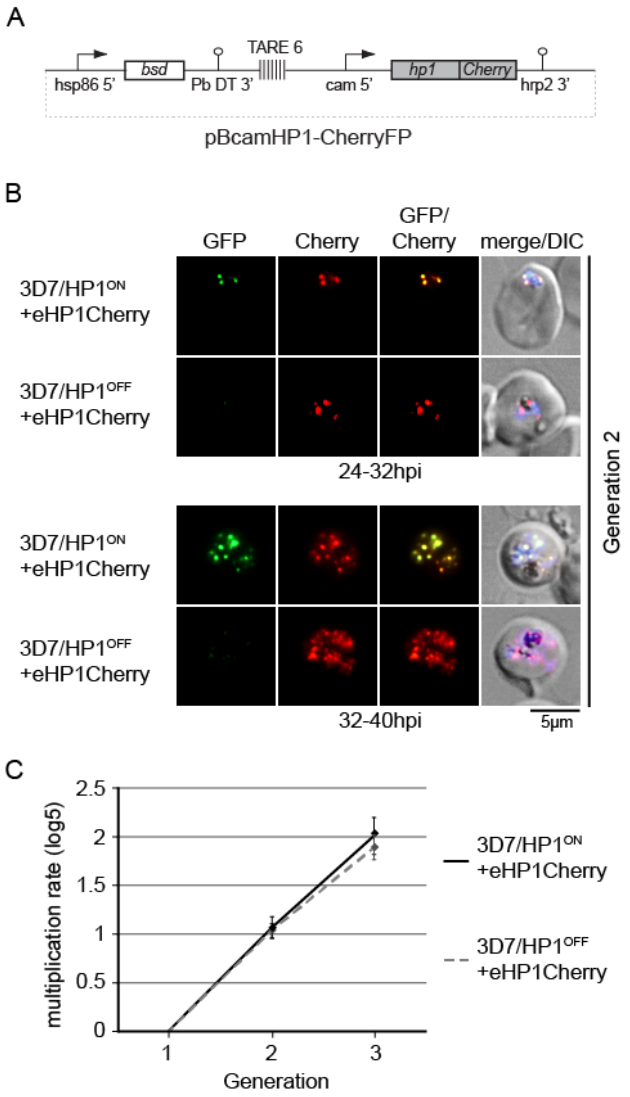


Figure S4

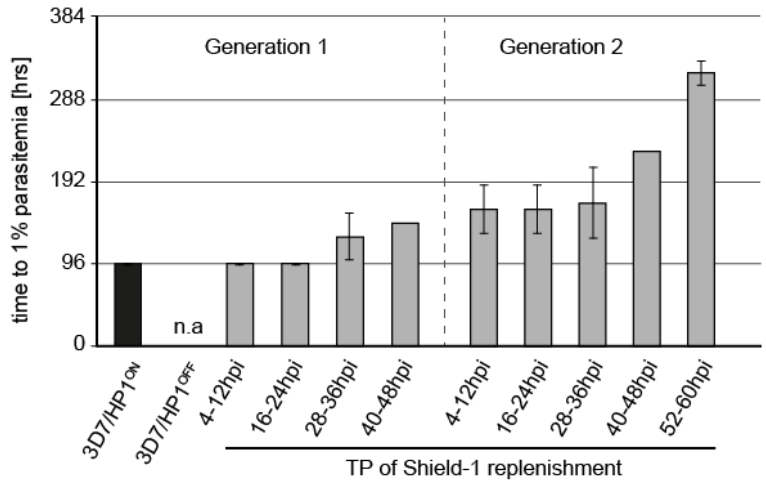


Figure S5

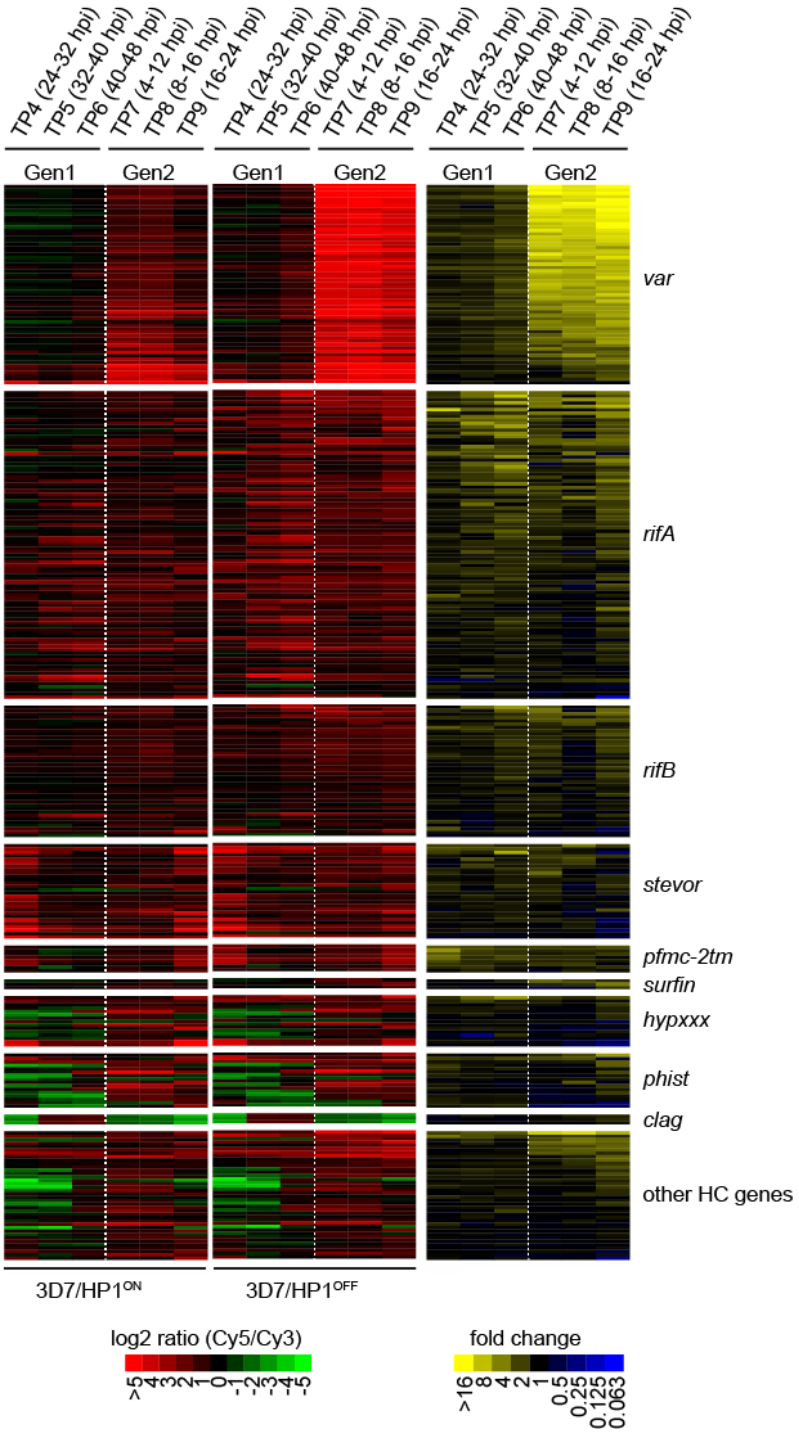

TP4 (24-32 hpi)
TP5 (32-40 hpi)
TP6 (40-48 hpi)
TP7 (4-12 hpi)
TP8 (8-16 hpi)
TP9 (16-24 hpi)
Gen1
Gen2
var
rifA
rifB
stevor
pfmc-2tm
surfin
hypxxx
phist
clag
other HC genes
3D7/HP1ON
3D7/HP1OFF
log2 ratio (Cy5/Cy3)
>5 4 3 2 1 0 -1 -2 -3 -4 -5
fold change
>16 8 4 2 1 0.5 0.25 0.125 0.063

Figure S6

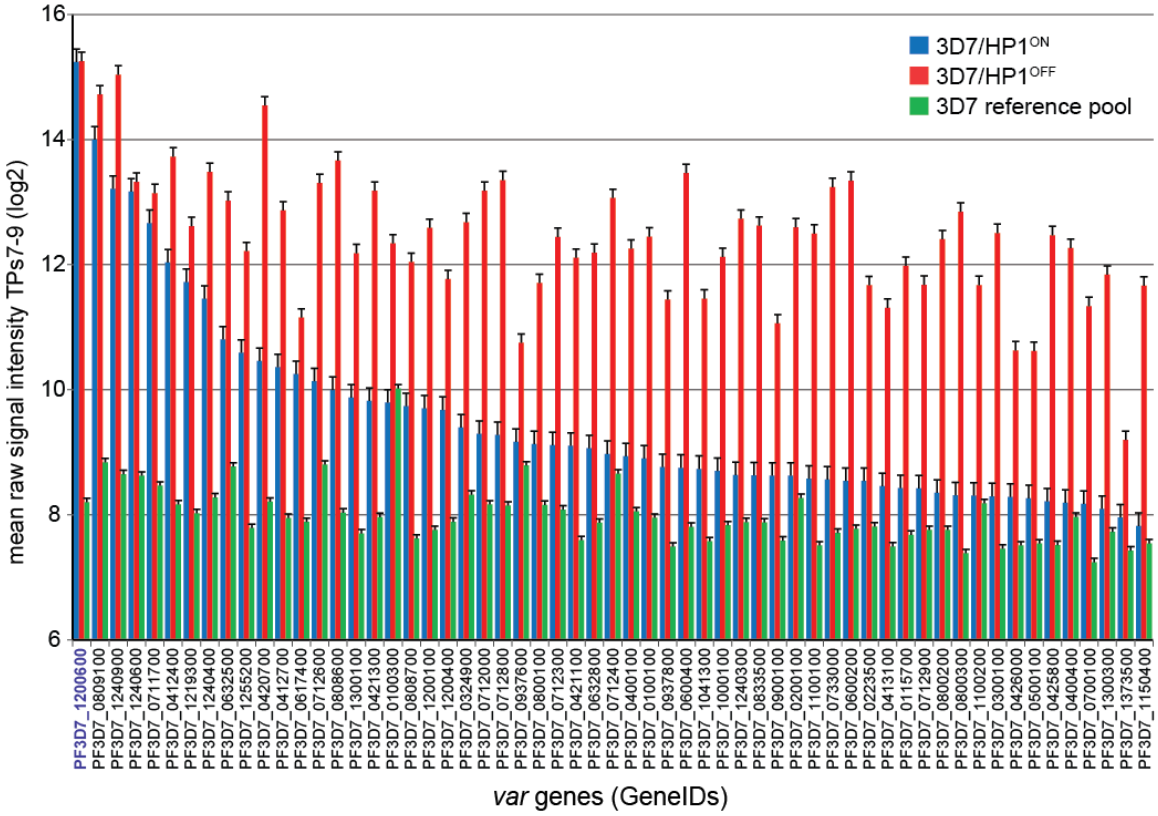


Figure S7

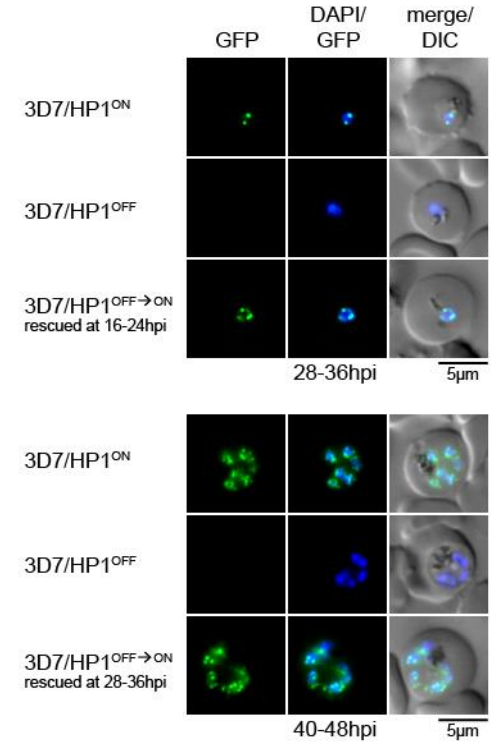


Figure S8

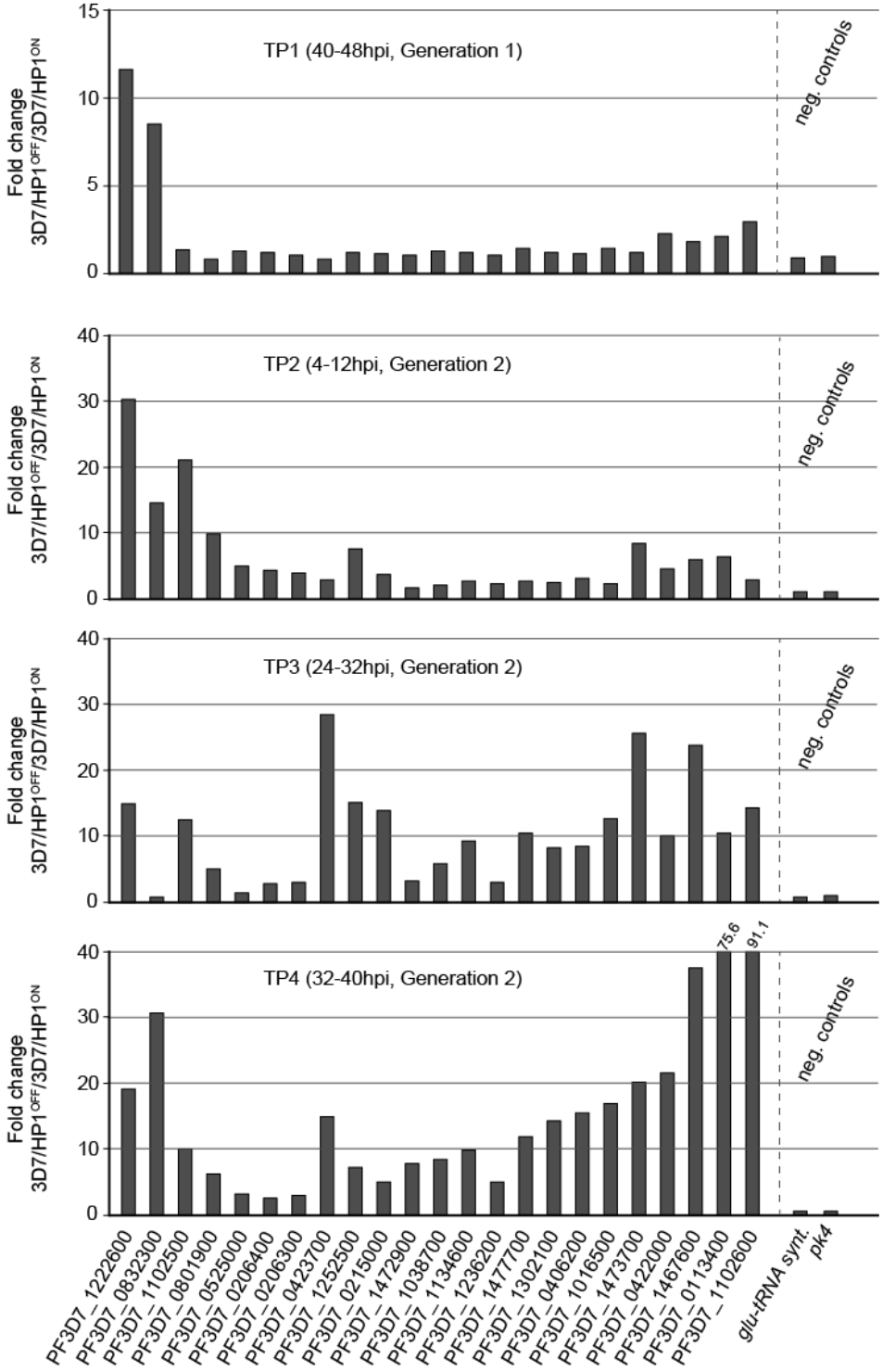
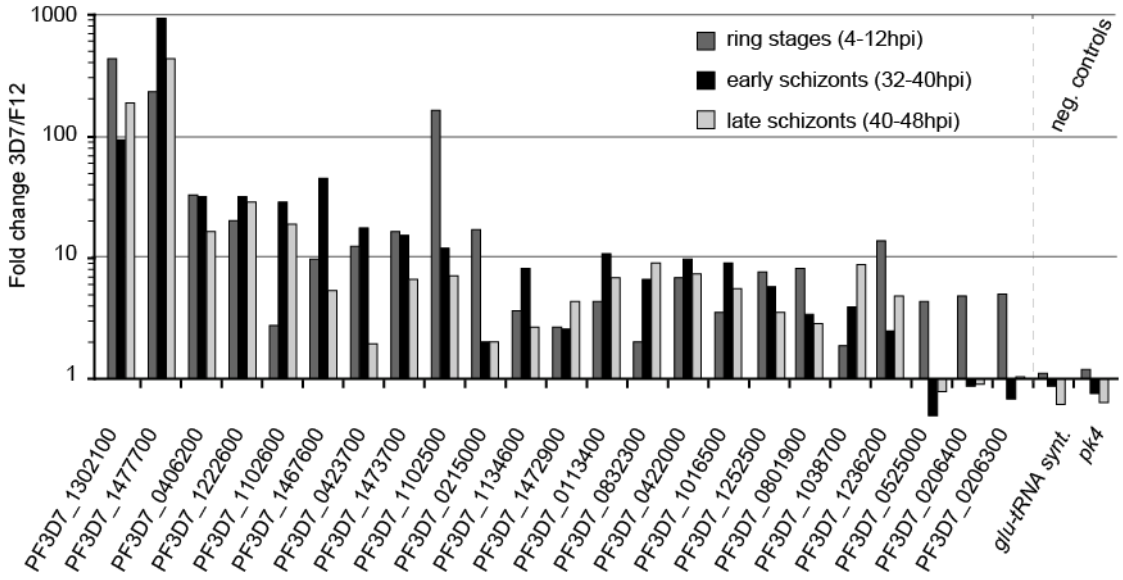

TP1 (40-48hpi, Generation 1)
TP2 (4-12hpi, Generation 2)
TP3 (24-32hpi, Generation 2)
TP4 (32-40hpi, Generation 2)
Fold change 3D7/HP1OFF/3D7/HP1ON
neg. controls
75.6
91.1
PF3D7_1222600
PF3D7_0832300
PF3D7_1102500
PF3D7_0801900
PF3D7_0525000
PF3D7_0206400
PF3D7_0206300
PF3D7_0423700
PF3D7_1252500
PF3D7_0215000
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

Figure S9


1000
100
10
1
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

5. General Discussion and Outlook

5.1 Singular Gene Choice

Phenotypic plasticity is essential for various biological processes and many organisms employ mutually exclusive expression of gene family members to equip individual cells with distinct features. In unicellular pathogens such as *Trypanosoma brucei* for instance, this phenomenon forms the basis for antigenic variation and facilitates immune evasion. Interestingly, higher eukaryotes like *Drosophila* and mice employ similar strategies for the establishment of their complex visual and olfactory systems. In the latter examples, individual cells must be equipped with distinct combinations or a single out of many sensory receptors in order to allow for the development of cellular networks with high discriminatory power. While mutually exclusive *var* gene expression in *P. falciparum* shares many regulatory characteristics with the abovementioned systems, in any case there are also prominent differences.

Silencing of variant surface glycoprotein (*vsg*) genes in *T. brucei* for example, in contrast to *var* gene repression, is independent of HP1. In fact, trypanosomes appear to lack HP1 altogether. However, as in the malaria parasite, transcriptional repression of telomere-proximal chromatin also involves the action of a histone deacetylase (SIR2rp1)[1]. Moreover, similar to the *var* expression site (VES) in *P. falciparum*, a nuclear body is associated with active *vsg* transcription. However, here mRNA is produced in a polycistronic manner by RNAPolI[2].

In *Drosophila*, different regulatory strategies are used to control genes coding for olfactory- (OR) and photo-receptors (Rh) in antennae and the retina, respectively. The five Rh members are almost exclusively controlled by a sophisticated interplay of specific TFs, resulting in the predefined arrangement of cells expressing a unique receptor or different Rh combinations[3]. TFs and their corresponding *cis*-acting elements also play a role in coordinating the expression of a single to few out of 62 OR genes in individual cells of the fly`s olfactory system. However, the control of OR loci additionally involves an epigenetic component[3]. More specifically, chromatin at repressed genes is associated with H3K9me2 and transcriptional activity could be linked to enriched levels of H3K4me3[4]. Additionally, and in contrast to singular PfEMP1 expression in the malaria parasite, OR choice in *Drosophila* involves a negative feedback that is provided by synthesised receptor proteins[3].

It is somewhat surprising that *P. falciparum var* gene regulation appears to share most features with mutually exclusive OR expression in the murine system. The OR family consists of more than 1200 genes and non-expressed OR family members cluster in few RNAPolII-depleted heterochromatic foci and are marked by H3K9me3 and HP1[5,6]. Moreover, reminiscent of the process that accompanies *var* activation, transcribed OR genes are no longer associated with these repressive sites and it was hypothesised that they are repositioned to a specialised, transcriptionally competent nuclear compartment[6]. In further analogy to *var* genes, Magklara and colleagues identified a functional association of H3K9me3 and H3K4me3 with repressed and transcriptionally active OR loci, respectively[5]. Additionally, each gene is provided with upstream sequence information that allows for its accurate participation in the process of OR gene choice[7–10]. Noteworthy, despite of the striking similarities compared to *var* genes, the control of murine ORs is characterised by at least one prominent difference as their expression was shown to involve a negative feedback on the protein level[11–14]. Moreover, activation of OR genes depends on the interaction with a unique enhancer sequence and several lines of evidence suggest that this H element does not only act in *cis* but also facilitates transcription of OR loci on other chromosomes[13,15,16]. Undoubtedly, the functional principle of the H element provides an attractive model for how a single *var* gene could be activated within the VES. However, attempts to identify similar interactions in *P. falciparum* failed[17] (Witmer, PhD Thesis 2011).

Although the above systems provided valuable information, we still lack an overall picture of the regulatory mechanisms that underlie mutually exclusive gene expression. Singular gene choice in different organisms may either have developed independently through convergent evolution, or alternatively may originate from a common regulatory concept. In either case, it is unlikely that this phenomenon obeys universal rules. The underlying gene regulatory principles, hence, have to be analysed separately.

In my PhD project, I used two complementary approaches to examine how *P. falciparum* parasites are able to establish and maintain singular *var* gene choice. First, a promoter mapping strategy was used to identify regulatory elements contained within *var ups* regions. Second, I aimed at a detailed functional characterisation of the epigenetic regulator PfHP1 by the generation of inducible loss-of-function mutants. Here, I would like to discuss a few aspects of my findings by focusing on subjects that have not already been mentioned in chapters 2 to 4.

5.2. *Cis*-Acting *ups* Elements

In a previous study, the *upsC* region of *var* gene PF3D7_1240600 was shown to be sufficient to infiltrate a drug selectable reporter into the programme of mutually exclusive *var* transcription[18]. Using a similar plasmid setup, we investigated truncated versions of this upstream sequence in search for *cis*-acting regulatory elements. This approach allowed us to assign singular *var* locus recognition to a 101bp element, the MEE, located downstream of the *upsC* TSS[19,20]. Noteworthy, the MEE sequence is bound by a nuclear factor that we termed MIF in a sequence-specific fashion. It is hence plausible that MIF is involved in linking the MEE to *trans*-acting activities that are essential for singular *var* gene choice.

Moreover, we could map two additional *var* gene features to distinct regulatory elements found within the *upsC* region. First, we found that the UAS, an activation sequence situated upstream of the TSS, is essential for inducing transcription at the promoter. Hypothetically, transcripts deriving from the upstream alternative transcriptional start site (aTSS) may contribute to UAS functionality. The exact role of this aTSS in *var* gene regulation, however, remains elusive. Second, in analogy to the post-transcriptional mechanisms that regulate *var2csa* expression, we observed a strong repressive effect of a 5`UTR element on translation of *upsC*-derived transcripts.

5.2.1. The UAS and Upstream Transcription Initiation

Uncoupling the UAS from the *upsC* promoter completely abolishes transcription. Conversely, when placed upstream of a heterologous promoter, this element is able to enhance transcriptional activity in both episomal and chromosomal contexts. These results unambiguously demonstrate that the UAS is an autonomous element that is most likely involved in the recruitment of sequence-specific transcriptional activators that eventually support RNAPolII-dependent transcription initiation. Interestingly, I found that alternative transcripts can be initiated from a position clearly upstream of the UAS element. Consequently, the UAS is surrounded by two transcriptional initiation sites on either side. Interestingly, this architecture is reminiscent of that found at the *fbp1*$^{+}$ locus in *S. pombe*. Here, two UAS elements, both of which are required for promoter activation[21], act in concert with aTSS sequences to mediate stepwise chromatin remodelling events that are essential for *fbp1*$^{+}$ induction[22]. More precisely, de-repression of this gene depends on passage of RNAPolII through the promoter region. This in turn is facilitated by UAS-mediated transcription initiation at the aTSS elements[22].

It will be interesting to test if similar mechanisms are operational in *var* gene regulation. Noteworthy, deletion of the *upsC* aTSS had no obvious effects on promoter activity. However, we may have missed a functional role of this upstream initiation site due the limited possibilities of investigating chromatin-related processes in an episomal context[23]. In analogy to the example of *fbp1*$^{+}$, it is plausible that endogenous *var* activation requires active transcription through the core promoter as a primer for subsequent chromatin remodelling events. Verifying this hypothesis, however, will be a challenging endeavour. In a first attempt I would recommend to introduce a transcriptional terminator downstream of both the aTSS and the UAS elements. If indeed upstream transcription is required for *var* promoter activation, parasites in which such engineered promoters drive expression of a drug-selectable promoter will be impaired in their ability to resist drug selection compared to a control population where the terminator sequence is placed in a non-functional position upstream of the aTSS. Given the success of this initial experiment, the *upsC* region would have to be systematically tested for the occurrence of additional transcript species as we have indication that further initiation sites exist (Brancucci, MSc Thesis 2008). Their exact role could then be assessed as indicated above. Similar to what was observed for *fbp1*$^{+}$ in *S. pombe*, it is conceivable that the *upsC* UAS (note that this element encompasses several hundred base pairs) is composed of two or more functional entities. If this is the case, it would be interesting to test whether and how they are involved in transcription initiation at both the main initiation site and the aTSS elements. In my opinion, the phenomenon of alternative transcriptional activity at *ups* regions would be an attractive avenue to pursue. The anticipated results may substantially contribute to our understanding of the logic behind *var* gene induction.

5.2.2. The MEE and Mutually Exclusive *var* Gene Activation

In absence of the MEE sequence, episomal *upsC* regions can be activated in addition to, rather than at the expense of endogenous *var* genes. Hence, unlike full length promoters, these constructs are able to escape mutually exclusive *var* locus recognition. Consistent with the current model of singular *var* gene choice, it is plausible that the MEE serves as a counting element with which silenced *var* members compete for unique de-repressive activities within the VES. The interaction of this element with the yet unknown nuclear factor MIF may link singular locus recognition to concomitant chromatin remodelling events that are essential for *var* promoter activation. In this context, it is important to mention that the MEE does not influence promoter activity. Mutually exclusive transcription and *var* promoter activation are

thus uncoupled processes. Nevertheless, the finding that the MEE reduces transcriptional activity in a heterologous context indicates that this DNA-protein interaction may still participate in *var* repression or silencing. A detailed model of how this may contribute to singular *var* gene choice is given in chapter 2. At the same time, it is conceivable that the binding of MIF may represent a prerequisite for VES-mediated *var* promoter activation. Clearly, efforts tailored towards the identification of this factor are now required. If successful, this would allow a functional characterisation of MIF using genetic deletion and/or conditional knock-down strategies. According to our preferred model we would expect that depletion of MIF causes relaxed silencing or, in the extreme case, even full activation of the entire *var* gene family. I am convinced that the anticipated results would significantly advance our knowledge on singular *var* gene choice. Unfortunately, my initial attempts to purify MIF were unsuccessful.

Further, in a complementary approach, it would be interesting to follow MEE-deprived *var* genes in their natural chromosomal context. This would require the targeted manipulation of *var* locus PF3D7_1240600. Replacing the MEE with a specific sequence tag would allow investigating effects of this element on *var* gene silencing. The inserted sequence may be used for parallel targeted chromatin immunoprecipitation and quantitative RT-PCR approaches. Like that, effects on promoter activity could be assigned to changes in chromatin composition. For instance, given a role of the MEE in *var* gene silencing, deletion of this element may result in transcriptional de-repression and the substitution of H3K9me3/PfHP1 and H3K36me3 with activating epigenetic marks. Importantly, it would be necessary to target an MEE proximal sequence using the same approach in order to control for effects that simply arise from genome manipulation. In a second step, substituting the PF3D7_1240600 coding sequence by a selectable marker would allow assessing the role of the MEE during *var* gene induction and active transcription.

Using electromobility shift assays we showed that MIF binds specifically to an 8bp motif within the *upsC* MEE and to semi-conserved sequence elements found across other *ups* types. Whereas this ATAGATTA sequence is essential for MIF-binding, presence of this motif alone is not sufficient to recruit MIF. Additional sequence information, hence, provides a striking specificity of this factor to *var* gene *ups* regions. This finding indicates that the MEE, irrespective of whether the interaction with MIF is involved in silencing or activation, has the potential to place all *var* genes under a common regulatory concept; a prerequisite for singular gene choice. In a likely scenario, this element provides access to a limiting factor within the VES that may subsequently activate *var* genes by interacting with histone-modifying enzymes

and chromatin remodelers, thereby releasing the *var* locus from heterochromatin-mediated silencing. The existence of a limiting factor has already been postulated by Dzikowski and Deitsch who showed that the presence of high numbers of constitutively active *var* promoters (uncoupled from the intron) represses endogenous *var* genes and completely erases the epigenetic memory of previously active members[24]. The authors hypothesised that this occurred because episomal promoters compete with the endogenous locus either for limited spatial affiliation with the VES or, alternatively for a VES-associated DNA element or protein complex. A follow-up study expanded this model to promoters of other virulence factors such as *rif* and *stevor*, indicating that several gene families, including the *vars*, share a common titratable factor essential for transcriptional activation[25]. The investigations of Witmer *et al.*, however, could not confirm such an inter-family relationship[26].

Although the details remain hitherto unknown, the MEE is certainly involved in mutually exclusive *var* gene expression. Further, the specific and subtype independent binding of MIF to *ups* regions render this unknown nuclear factor a prime candidate for the recruitment of the underlying regulatory activities. Detectability of this interaction in electromobility shift assays indicates that MIF is expressed at moderate to high levels. Hence, this protein may represent a bridging factor rather than the actual limiting component of the VES. Hypothetically, MIF or a MIF-containing protein complex may recruit a formerly silenced locus to a unique *trans*-acting enhancer that, in analogy to H element-dependent OR gene choice[15], allows for transcriptional activity. Noteworthy, neither the ATAGATTA motif nor any other sequences found within the 101 base pair MEE are predicted binding sites of ApiAP2 proteins[27], suggesting that MIF belongs to a yet unknown class of TFs in *P. falciparum*.

5.3. Lessons Learned from PfHP1

Using a conditional loss-of-function approach based on the DD/Shield-1 system[28], we uncovered essential roles for PfHP1 in virulence gene expression and cell cycle progression. In the generation following PfHP1-depletion, parasites display hyper-activity of virulence genes and the breakdown of mutually exclusive *var* expression before they eventually enter a complete cell cycle arrest at the G1/S transition phase. Continued culturing revealed that PfHP1-deprived parasites can be subdivided into two prominent phenotypic groups. Approximately half of these cells entered a state of dormancy, whereas the other half were committed to sexual differentiation and completed gametocytogenesis in absence of PfHP1. Hence, these results demonstrate for the first time that sexual differentiation of malaria parasites involves a strong epigenetic component.

5.3.1. *var* Gene Silencing

Among over 400 genes that are PfHP1-demarcated[29], the *var* gene family reacts most sensitively to reduced levels of this epigenetic factor. After removing Shield-1 during the early phase of the IDC, PfHP1 physically dissociates from chromatin prior to 40-48 hpi. In comparison, the hyper-activity of *var* genes is delayed and becomes significant only during ring stage development of the following generation. These results demonstrate that *var* members retain their stage-specific expression profile also in absence of PfHP1. In line with the essential role of the UAS in promoter activation, our observations strongly suggest that specific TFs are indispensable for active *var* transcription and it will be interesting to test if this achieved by a common (set of) *trans*-acting factor(s) or in a subgroup- or even gene-specific manner.

Our current efforts aim at investigating the role of PfHP1 in maintaining epigenetic memory at *var* loci. More specifically, we will test whether temporary PfHP1-depletion affects *var* gene regulation in subsequent generations. We will allow depleted parasites to re-accumulate PfHP1 shortly before the cells enter cell cycle arrest. Note that immediately prior to the time point of rescue, all *var* gene promoters are activated. During the subsequent S- and M-phases, where *var* genes are transcriptionally repressed, newly synthesised PfHP1 will be available to repopulate *var* loci. However, it remains to be seen whether PfHP1 will be recruited immediately to chromatin. Under wild type conditions, active transcription is required to mark *var* genes for re-activation in the following cell cycle[24]. It is thus conceivable that in rescued PfHP1-depleted parasites each member of the *var* gene family remains in a poised state, which may compete with PfHP1-mediated silencing. Western blot and IFA techniques will be used to test if indeed re-establishment of *var* gene silencing is impaired in this situation. Importantly, antibodies specific to individual PfEMP1 variants will allow to analyse *var* gene activity on the single cell level (these antibodies have been kindly provided by T. Lavstsen, University of Copenhagen). In the case that mutually exclusive transcription is not immediately re-established, it will be interesting to investigate chromatin composition at the respective *var* loci. If on the other hand *var* gene silencing is readily re-established, we could tackle the important question of whether mutually exclusive transcription reverts back to the previously active *var* member or not and thus unravel the role of PfHP1 in transgenerational inheritance. Depending on the observed effects, either ChIP-qPCR or ChIP-Seq approaches will be favourable to investigate the chromatin landscape at *var* gene loci. In any case, I am confident that these experiments will provide us with novel

insights into the dynamics and the mechanisms that underlie PfHP1-recruitment to chromatin and the maintenance of epigenetic memory in *P. falciparum*.

5.3.2. Silencing of Other Clonally Variant Factors

In addition to *var* genes, many members of other heterochromatic virulence gene families, including *rif*, *stevor* and *pfmc-2tm*, are up-regulated upon PfHP1-depletion. In contrast to the complete de-repression of *var*s, however, the effects are less prominent in most cases and a significant dysregulation is restricted to individual family members only. Similar to what we observed for the *var* family, these genes appear to retain their stage-specific expression profile in absence of PfHP1. Noteworthy, peak transcription of many of these genes is not reached by the time point at which PfHP1-depleted parasites enter the cell cycle arrest[30–32]. It is hence likely that we missed a more pronounced up-regulation of these gene families simply due to the temporal constraints of our transcriptome analysis. This view is supported by the fact that even among the non-significantly dysregulated genes, the majority is still de-repressed.

Clonally variant expression was also reported for factors involved in nutrient uptake and erythrocyte invasion[33–36]. Interestingly, four of these proteins are encoded by genes for which the active and silenced state has been functionally linked to the presence of H3K9ac and H3K9me3 marks, respectively[37–39], suggesting that also here PfHP1 is involved in transcriptional regulation. Reminiscent of mutually exclusive *var* activity, only one of two *clag3* genes, *clag3.1* and *clag3.2* respectively, is transcriptionally active per parasite[36–38]. While the *clag3* variants, coding for a surface anion channel constituent[40], are heterochromatic, the invasion genes *eba-140* and *rh4*[41] lie within euchromatic chromosomal domains[29]. Interestingly, despite of their association with H3K9me3, none of these genes showed a significant response to PfHP1-depletion in our experiment. Whereas it is conceivable that *eba-140* and *rh4* have already been active prior to the loss of PfHP1 function, this result is particularly surprising with respect to the *clag3* genes. Since our transcriptional profiling was carried out using a clonal parasite line, we expected one of the two genes to be silenced on the population level. Chromatin immunoprecipitation revealed that indeed PfHP1 was greatly enriched at the *clag3.2* locus compared to *clag3.1* in 3D7/HP1^{ON} parasites, suggesting that this variant was epigenetically silenced (Figure 1). However, we did not observe de-repression of this gene in 3D7/HP1^{OFF} cells despite the marked reduction in PfHP1 occupancy. Further, the tandem arrangement of the two *clag3* genes rules out the possibility that *clag3.2* localises to a different nuclear compartment with

restricted availability to TFs compared to *clag3.1*. Therefore, I consider it likely that PfHP1-independent processes are involved in the regulation of mutually exclusive *clag3* gene expression. These may include negative feedback signals on either the protein or transcript level and it will be interesting to assay the underlying regulatory strategies.

Figure 1

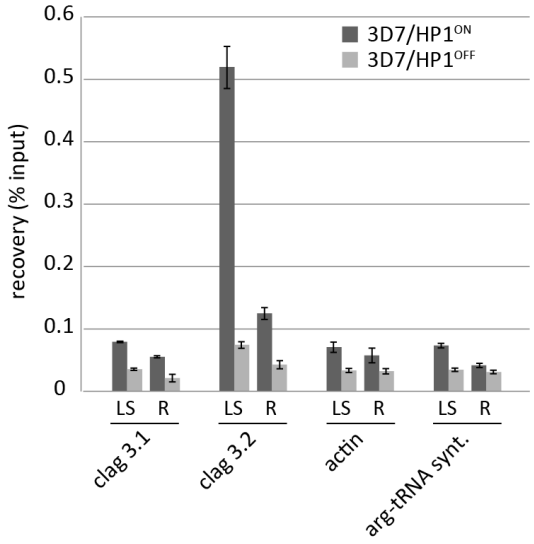


Figure 1. PfHP1 occupancy at the *clag3* genes and two euchromatic control loci. Shield was removed at 4-12hpi in generation 1. LS, late schizont stage (48-48hpi in generation 1); R, ring stage (16-24hpi in generation 2). αPfHP1 antibodies were used for ChIP. Values represent the mean of three technical replicates (+/- SD). αIgG values (background) have been subtracted. The ChIP protocol is given in the Extended Experimental Procedures section of chapter 4.

5.3.3. AP2-G and Sexual Differentiation

Our comparative transcriptional profiling demonstrated that the high rate at which PfHP1-depleted parasites enter sexual differentiation is linked to the de-repression of *ap2-g*, the only PfHP1-demarcated *apiap2* family member[29]. Work in the Llinás and Waters laboratories revealed that indeed this factor is essential for sexual conversion as null mutants of both *P. falciparum* and the rodent malaria parasite *P. berghei* are incapable of producing gametocytes[42,43]. The observation that expression of AP2-G, which is induced at 34-40 hpi, is followed by an orchestrated upregulation of gametocyte-specific genes suggests that this TF directly or indirectly initiates a gene cascade designated to sexual development. Hence, PfHP1 represents a key component of the regulatory switch that balances asexual growth against gametocyte formation through the control of *ap2-g* activity.

ap2-g belongs to a small group of genes that, despite of their association with PfHP1, lie within euchromatic domains. In model organisms, the binding of HP1 to such loci can correlate with transcriptional repression[44–46]. The findings of Ayyanathan and colleagues provides an interesting blueprint for how a euchromatic locus can be specifically silenced in an HP1-dependent manner[47]. The authors used an inducible system to target murine KAP1, a KRAB co-repressor, to the promoter of a highly transcribed transgene. Through the recruitment of histone-modifying enzymes and HP1[48–50], KAP1 was shown to coordinate spatially restricted chromatin remodelling events that eventually result in the heritable silencing of gene activity. Hypothetically, such KAP1-HP1-mediated silencing may be

targeted to specific euchromatic loci via KRAB domain-containing TFs[51]. Although neither KRAB-proteins nor KAP1 have homologs in malaria parasites, it will be interesting to test whether conceptually similar mechanisms are employed by *P. falciparum* in order to recruit PfHP1 to *ap2-g* and other euchromatic target loci. The use of PfHP1 in mediating transcriptional silencing outside of heterochromatin, however, appears to be very restricted. In fact, besides *ap2-g* we observed only one additional euchromatic gene, *msp3.8* (PF3D7_1036300), significantly up-regulated in 3D7/HP1^{OFF} cells.

Sexual differentiation is a prerequisite for the development of parasites within the mosquito vector and is thus essential for transmission. However, gametocyte formation is paradoxical in the sense that this irreversible cell fate decision antagonises asexual replication and hence limits its own source. Therefore, the balance between asexual replication and sexual development needs to be tightly controlled. The rate of sexual conversion varies between parasite strains, both *in vivo* and *in vitro*[52–54]. Whereas this implies the involvement of parasite-intrinsic factors, genetic host cell variations are also known to affect sexual parasite differentiation. Specifically, dysregulation of certain micro RNA (miRNA) species in host cells has been linked to translational inhibition of specific parasite transcripts and concomitant gametocyte formation[55]. Interestingly, two recent studies provide a potential connection between this observation and the controlled de-repression of *ap2-g*. Mantel *et al.* and Regev-Rudzki *et al.* demonstrated that infected erythrocytes release microvesicles that are targeted to other parasites in the population[56,57]. Intriguingly, this system allows for the lateral transfer of DNA[57] and both studies independently reported increased gametocyte formation in recipient cells. It is thus tempting to speculate that microvesicle-uptake triggers a pathway that feeds into the AP2-G-mediated activation of early gametocyte-specific genes, either by directly modulating PfHP1-controlled *ap2-g* transcription, or by activating effectors that in turn regulate this epigenetic switch. Incorporated host miRNA species may for instance inhibit the synthesis of KAP1/KRAB-like repressor proteins in *P. falciparum* and thereby specifically release *ap2-g* from PfHP1-mediated silencing.

In an alternative, albeit not mutually exclusive hypothesis, the *ap2-g* promoter may participate in the programme of singular *var* gene choice. In this very speculative scenario, switches in transcriptional *var* gene activity would control the rate at which parasites differentiate into gametocytes. If this is the case, the 5` region of *ap2-g*, similar to a full length *var* promoter, must be able to interfere with active *var* transcription. Using the same approach as described for the mapping of *cis*-acting regulatory elements in the *ups* region of

var genes (chapter 2), one could test whether indeed activity of the *ap2-g* promoter is able to silence *var* gene transcription.

Interestingly, our data suggests that AP2-G is the only transcriptional regulator whose expression is under epigenetic control during intra-erythrocytic parasite development. This may reflect the prominent role of this TF in mediating the escape from an otherwise pre-defined transcriptional programme dedicated to interminable asexual replication. A comparable cell cycle exit was observed for liver stage parasites of *P. vivax*. Prior to schizogony within hepatocytes, sporozoites develop into dormant hypnozoites that, during a later time point, may cause the relapse of symptomatic infections[58]. It will be very interesting to investigate whether the observed G1-arrest in *P. falciparum* shares regulatory features with this phenomenon. If this is the case, PfHP1-depleted parasites may become an invaluable model for these dormant stages of malaria parasites. Unfortunately, however, such studies may be impossible to be conducted until *P. vivax* in vitro culture systems become available.

5.4. Future Directions

Singular *var* gene choice represents an attractive target for future anti-malarial therapies. Interfering with VES-mediated transcriptional activation of *var* genes may be used to inhibit morbidity and mortality caused by cytoadherent *P. falciparum* parasites. However, to reach this goal it will be necessary to gain further information on the underlying regulatory strategies. In my opinion, the identified *cis*-acting *var* promoter elements and MIF should be prioritised in such efforts.

We showed that depletion of PfHP1 forces more than half of parasites in an *in vitro*-cultured population to enter the sexual pathway. The conditional loss-of-function system allows for the production of gametocytes with high purity and synchronicity. Hence, these parasites may be used as a tool for investigating questions regarding both sexual commitment and gametocyte development. The importance of such information is underpinned by the fact that transmission reduction has become a central building block in global efforts to control and eradicate malaria. Knowledge on how malaria parasites regulate sexual stage conversion may provide the basis for future transmission interventions. Last but not least, gametocytes induced by the loss of PfHP1-function may become valuable in high-throughput drug screening assays and thereby essentially contribute to the development of gametocyticidal medication. I am both confident and hopeful that our findings will contribute to the development of novel and effective malaria intervention strategies.

5.5. References

1. Alsford, S., Kawahara, T., Isamah, C. & Horn, D. A sirtuin in the African trypanosome is involved in both DNA repair and telomeric gene silencing but is not required for antigenic variation. *Mol. Microbiol.* **63,** 724–736 (2007).
2. Navarro, M. & Gull, K. A pol I transcriptional body associated with VSG mono-allelic expression in Trypanosoma brucei. *Nature* **414,** 759–763 (2001).
3. Rister, J., Desplan, C. & Vasiliauskas, D. Establishing and maintaining gene expression patterns: insights from sensory receptor patterning. *Dev. Camb. Engl.* **140,** 493–503 (2013).
4. Sim, C. K., Perry, S., Tharadra, S. K., Lipsick, J. S. & Ray, A. Epigenetic regulation of olfactory receptor gene expression by the Myb-MuvB/dREAM complex. *Genes Dev.* **26,** 2483–2498 (2012).
5. Magklara, A. *et al.* An epigenetic signature for monoallelic olfactory receptor expression. *Cell* **145,** 555–570 (2011).
6. Clowney, E. J. *et al.* Nuclear aggregation of olfactory receptor genes governs their monogenic expression. *Cell* **151,** 724–737 (2012).
7. Qasba, P. & Reed, R. R. Tissue and zonal-specific expression of an olfactory receptor transgene. *J. Neurosci. Off. J. Soc. Neurosci.* **18,** 227–236 (1998).
8. Vassalli, A., Rothman, A., Feinstein, P., Zapotocky, M. & Mombaerts, P. Minigenes impart odorant receptor-specific axon guidance in the olfactory bulb. *Neuron* **35,** 681–696 (2002).
9. Bozza, T. *et al.* Mapping of class I and class II odorant receptors to glomerular domains by two distinct types of olfactory sensory neurons in the mouse. *Neuron* **61,** 220–233 (2009).
10. Khan, M., Vaes, E. & Mombaerts, P. Regulation of the probability of mouse odorant receptor gene choice. *Cell* **147,** 907–921 (2011).
11. Shykind, B. M. *et al.* Gene switching and the stability of odorant receptor gene choice. *Cell* **117,** 801–815 (2004).
12. Lewcock, J. W. & Reed, R. R. A feedback mechanism regulates monoallelic odorant receptor expression. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 1069–1074 (2004).
13. Serizawa, S. *et al.* Negative feedback regulation ensures the one receptor-one olfactory neuron rule in mouse. *Science* **302,** 2088–2094 (2003).
14. Nguyen, M. Q., Zhou, Z., Marks, C. A., Ryba, N. J. P. & Belluscio, L. Prominent roles for odorant receptor coding sequences in allelic exclusion. *Cell* **131,** 1009–1017 (2007).
15. Lomvardas, S. *et al.* Interchromosomal interactions and olfactory receptor choice. *Cell* **126,** 403–413 (2006).
16. Fuss, S. H., Omura, M. & Mombaerts, P. Local and cis effects of the H element on expression of odorant receptor genes in mouse. *Cell* **130,** 373–384 (2007).
17. Lemieux, J. E. *et al.* Genome-wide profiling of chromosome interactions in Plasmodium falciparum characterizes nuclear architecture and reconfigurations associated with antigenic variation. *Mol. Microbiol.* (2013). doi:10.1111/mmi.12381
18. Voss, T. S. *et al.* A var gene promoter controls allelic exclusion of virulence genes in Plasmodium falciparum malaria. *Nature* **439,** 1004–1008 (2006).
19. Deitsch, K. W., del Pinal, A. & Wellems, T. E. Intra-cluster recombination and var transcription switches in the antigenic variation of Plasmodium falciparum. *Mol. Biochem. Parasitol.* **101,** 107–116 (1999).
20. Voss, T. S. *et al.* Genomic distribution and functional characterisation of two distinct and conserved Plasmodium falciparum var gene 5’ flanking sequences. *Mol. Biochem. Parasitol.* **107,** 103–115 (2000).

21. Neely, L. A. & Hoffman, C. S. Protein kinase A and mitogen-activated protein kinase pathways antagonistically regulate fission yeast fbp1 transcription by employing different modes of action at two upstream activation sites. *Mol. Cell. Biol.* **20,** 6426–6434 (2000).
22. Hirota, K. *et al.* Stepwise chromatin remodelling by a cascade of transcription initiation of non-coding RNAs. *Nature* **456,** 130–134 (2008).
23. Horrocks, P. & Lanzer, M. Differences in nucleosome organization over episomally located plasmids coincides with aberrant promoter activity in P. falciparum. *Parasitol. Int.* **48,** 55–61 (1999).
24. Dzikowski, R. & Deitsch, K. W. Active transcription is required for maintenance of epigenetic memory in the malaria parasite Plasmodium falciparum. *J. Mol. Biol.* **382,** 288–297 (2008).
25. Howitt, C. A. *et al.* Clonally variant gene families in Plasmodium falciparum share a common activation factor. *Mol. Microbiol.* **73,** 1171–1185 (2009).
26. Witmer, K. *et al.* Analysis of subtelomeric virulence gene families in Plasmodium falciparum by comparative transcriptional profiling. *Mol. Microbiol.* **84,** 243–259 (2012).
27. Campbell, T. L., De Silva, E. K., Olszewski, K. L., Elemento, O. & Llinás, M. Identification and genome-wide prediction of DNA binding specificities for the ApiAP2 family of regulators from the malaria parasite. *PLoS Pathog.* **6,** e1001165 (2010).
28. Armstrong, C. M. & Goldberg, D. E. An FKBP destabilization domain modulates protein levels in Plasmodium falciparum. *Nat. Methods* **4,** 1007–1009 (2007).
29. Flueck, C. *et al.* Plasmodium falciparum heterochromatin protein 1 marks genomic loci linked to phenotypic variation of exported virulence factors. *PLoS Pathog.* **5,** e1000569 (2009).
30. Kyes, S. A., Rowe, J. A., Kriek, N. & Newbold, C. I. Rifins: a second family of clonally variant proteins expressed on the surface of red cells infected with Plasmodium falciparum. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 9333–9338 (1999).
31. Kaviratne, M., Khan, S. M., Jarra, W. & Preiser, P. R. Small variant STEVOR antigen is uniquely located within Maurer's clefts in Plasmodium falciparum-infected red blood cells. *Eukaryot. Cell* **1,** 926–935 (2002).
32. Sam-Yellowe, T. Y. *et al.* A Plasmodium gene family encoding Maurer's cleft membrane proteins: structural properties and expression profiling. *Genome Res.* **14,** 1052–1059 (2004).
33. Stubbs, J. *et al.* Molecular mechanism for switching of P. falciparum invasion pathways into human erythrocytes. *Science* **309,** 1384–1387 (2005).
34. Nery, S. *et al.* Expression of Plasmodium falciparum genes involved in erythrocyte invasion varies among isolates cultured directly from patients. *Mol. Biochem. Parasitol.* **149,** 208–215 (2006).
35. Gaur, D. *et al.* Upregulation of expression of the reticulocyte homology gene 4 in the Plasmodium falciparum clone Dd2 is associated with a switch in the erythrocyte invasion pathway. *Mol. Biochem. Parasitol.* **145,** 205–215 (2006).
36. Cortés, A. *et al.* Epigenetic silencing of Plasmodium falciparum genes linked to erythrocyte invasion. *PLoS Pathog.* **3,** e107 (2007).
37. Crowley, V. M., Rovira-Graells, N., Ribas de Pouplana, L. & Cortés, A. Heterochromatin formation in bistable chromatin domains controls the epigenetic repression of clonally variant Plasmodium falciparum genes linked to erythrocyte invasion. *Mol. Microbiol.* **80,** 391–406 (2011).
38. Comeaux, C. A., Coleman, B. I., Bei, A. K., Whitehurst, N. & Duraisingh, M. T. Functional analysis of epigenetic regulation of tandem RhopH1/clag genes reveals a role in Plasmodium falciparum growth. *Mol. Microbiol.* **80,** 378–390 (2011).

39. Jiang, L. *et al.* Epigenetic control of the variable expression of a Plasmodium falciparum receptor protein for erythrocyte invasion. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 2224–2229 (2010).
40. Nguitragool, W. *et al.* Malaria parasite clag3 genes determine channel-mediated nutrient uptake by infected red blood cells. *Cell* **145,** 665–677 (2011).
41. Cowman, A. F. & Crabb, B. S. Invasion of red blood cells by malaria parasites. *Cell* **124,** 755–766 (2006).
42. Llinas, M. Sex or no Sex? That is the question! Presented at MAM meeting in Lorne, Australia. (2012).
43. Waters, A. P. Identification of a conserved protein that is central to gametocytogenesis in Plasmodium berghei. Presented at MAM meeting in Lorne, Australia. (2012).
44. James, T. C. *et al.* Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of Drosophila. *Eur. J. Cell Biol.* **50,** 170–180 (1989).
45. Fanti, L., Berloco, M., Piacentini, L. & Pimpinelli, S. Chromosomal distribution of heterochromatin protein 1 (HP1) in Drosophila: a cytological map of euchromatic HP1 binding sites. *Genetica* **117,** 135–147 (2003).
46. Grewal, S. I. S. & Moazed, D. Heterochromatin and epigenetic control of gene expression. *Science* **301,** 798–802 (2003).
47. Ayyanathan, K. *et al.* Regulated recruitment of HP1 to a euchromatic gene induces mitotically heritable, epigenetic gene silencing: a mammalian cell culture model of gene variegation. *Genes Dev.* **17,** 1855–1869 (2003).
48. Schultz, D. C., Friedman, J. R. & Rauscher, F. J., 3rd. Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. *Genes Dev.* **15,** 428–443 (2001).
49. Schultz, D. C., Ayyanathan, K., Negorev, D., Maul, G. G. & Rauscher, F. J., 3rd. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes Dev.* **16,** 919–932 (2002).
50. Lechner, M. S., Begg, G. E., Speicher, D. W. & Rauscher, F. J., 3rd. Molecular determinants for targeting heterochromatin protein 1-mediated gene silencing: direct chromoshadow domain-KAP-1 corepressor interaction is essential. *Mol. Cell. Biol.* **20,** 6449–6465 (2000).
51. Urrutia, R. KRAB-containing zinc-finger repressor proteins. *Genome Biol.* **4,** 231 (2003).
52. Graves, P. M., Carter, R. & McNeill, K. M. Gametocyte production in cloned lines of Plasmodium falciparum. *Am. J. Trop. Med. Hyg.* **33,** 1045–1050 (1984).
53. Dyer, M. & Day, K. P. Commitment to gametocytogenesis in Plasmodium falciparum. *Parasitol. Today Pers. Ed* **16,** 102–107 (2000).
54. Abdel-Wahab, A. *et al.* Dynamics of gametocytes among Plasmodium falciparum clones in natural infections in an area of highly seasonal transmission. *J. Infect. Dis.* **185,** 1838–1842 (2002).
55. LaMonte, G. *et al.* Translocation of sickle cell erythrocyte microRNAs into Plasmodium falciparum inhibits parasite translation and contributes to malaria resistance. *Cell Host Microbe* **12,** 187–199 (2012).
56. Mantel, P.-Y. *et al.* Malaria-infected erythrocyte-derived microvesicles mediate cellular communication within the parasite population and with the host immune system. *Cell Host Microbe* **13,** 521–534 (2013).
57. Regev-Rudzki, N. *et al.* Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. *Cell* **153,** 1120–1133 (2013).
58. Cogswell, F. B. The hypnozoite and relapse in primate malaria. *Clin. Microbiol. Rev.* **5,** 26–35 (1992).

Personal Acknowledgements

First of all, I would like to thank Till Voss for (1) the confidence he has placed in me throughout these years, (2) his great supervision, (3) the hands-on support in wet-lab and office and (4) for teaching me how to summarise things with the help of a few numbers and brackets ☺. I was certainly right when writing in my master thesis that "his passion for malaria research is more infective than any *P. falciparum* sporozoite will ever be". Thank you for being so infective!

Further, I highly appreciate that Kirk Deitsch is part of my thesis committee and thank him for taking the long trip from New York to Basel!

I would also like to thank all the former and present GR group members. Especially Olivier Dietz, not only for sharing the good moments during our studies and free time but also for halving the worries we both had from time to time. Our joint first day in the GR lab would probably have been different, however, not less exciting if Kathrin (KW, Kiwi, Schnalz) Witmer was already back from Australia at that time. In this sense, I would like to thank KW for the amazing time we had in the lab as well as outside. I loved sharing the search for *cis*-acting *var* promoter elements with you! – Mostly because of but, as you know, sometimes also despite of the project that may be described with Edward Lorenz` words about a common phenomenon in nature: "Chaos: When the present determines the future, but the approximate present does not approximately determine the future" (Danforth, C. M., 2013). Certainly, the work on PfHP1 was more straightforward, albeit not less work-intensive. In particular, I would like to thank Nicole Bertschi, not only for pushing our joint project forward but also for all the great moments out there on the climbing walls! I also thank Christian (Chrigu) Flück for his help and cheerful attitude; we still miss you and KW as great members of the GR family! Moreover, I am grateful that I had the opportunity to work together with people who created a lovely and fun atmosphere in the lab, namely and in alphabetical order Andreas Pulfer, Cheryl Gysel, Elvis Ajuh, Hai Bui, Igor Niederwieser, Johanna Wetzel, Michael Filarsky, Sophie Oehring and Stefan Schneitter. You are great!

Many thanks also go to all Molecular Parasitology and Molecular Diagnostics group members. It was great having such smart and dynamic persons as you Esther Mundwiler and Hans-Peter Beck as fellows in science and on the unihockey and squash courts, respectively. Natalie Hofmann and Rahel Wampfler, the two angels from next door, shall not be missing here; thank you for all the inspiring conversations in and outside the lab! Not to forget the third angel, Serej Ley, either situated up there near the clouds on the second floor of the Swiss

TPH or in Papua New-Guinea. Further I like to thank, Ingrid Felger, Sebastian Rusch, Alex Oberli, Martin Maire and Françoise Brand who are able turn the cellar of the Swiss TPH into the same pleasant place it was already before Caroline Kulangara, Anna Perchuc, Dania Müller and Christian Köpfli left.

Warm thanks also go to other present and former members of the institute who created and still create a lovely atmosphere. Among many others, these are: Christoph Schmid, Reto Brun, Matthias Rottmann, Pascal Mäser, Sergio Wittlin, Pie Müller, Christian Scheurer, Benjamin Speich, Noemi Hiroshige, Urs Duthaler, Kathrin Ingram, Lucienne Tritten, Yvette Endriss, Pascale Steiger, Karin Gysin and Antoine Bruchlen.

Moreover, I like to thank the Boehringer Ingelheim Foundation for the financial support. Noteworthy, I highly appreciate the work of the people behind this foundation who, very importantly, do also support the BIF-family members with respect to their personal development.

Last but not least, I would like to thank you, Heidi Brancucci and Susanne Lüdi, for believing in me and for your support in every situation of life. Merci!

Reference

Danforth, C. M. Chaos in an Atmosphere Hanging on a Wall. *Math. Planet Earth 2013* (2013).

CURRICULUM VITAE

Surname:	Brancucci	Phone:	+41 76 507 30 88
First Names:	Nicolas Michel Beat	E-mail:	nicolas.brancucci@unibas.ch
Nationality:	Swiss	Date of birth:	31st October 1982
Address:	CH-4054 Basel, Bristenweg 20	Place of birth:	Basel, Switzerland

Education / Employments

4/2009-11/2013	PhD thesis at the Swiss Tropical and Public Health Institute, University of Basel, Switzerland, supervised by Prof. Till Voss Title: Novel Insights into the Epigenetic Regulation of *Plasmodium falciparum* Virulence Genes
2/2009	Master of Science in Infection Biology and Epidemiology at the Swiss Tropical Institute, University of Basel, Switzerland, supervised by Prof. Till Voss, grade 6.0
7/2007	Bachelor of Science in Biology, Major in Integrative Biology, grade 5.4 Undergraduate studies in Biology, University of Basel, Switzerland
7/2003-9/2004	Employment as an assistant meeting organiser in Actelion Pharmaceuticals Ltd., Allschwil, Switzerland
6/2003	Matura Typus E, Economics and Law, grade 5.25 Wirtschaftsgymnasium Basel, Switzerland

Further Education

6/2012	Bioinformatics training on the „Analysis of differential gene expression", Swiss Institute of Bioinformatics, Lausanne, Switzerland

Scholarship

12/2009-4/2012	PhD thesis was funded by the „Boehringer Ingelheim Fonds – Stiftung für medizinische Grundlagenforschung"

Conference Participation

5/2013	Oral presentation, 9th Annual BioMalPar Conference, Heidelberg, Germany, „Depletion of heterochromatin protein 1 in malaria parasites triggers hyper-expression of virulence genes, cell cycle arrest and sexual conversion"
9/2012	Poster presentation, 23rd Molecular Parasitology Meeting, Woods Hole, MA, USA
5/2012	Short talk and poster presentation, 8th Annual BioMalPar Conference, Heidelberg, Germany, „Exclude and Repress – A dual role for 5` upstream regions in *Plasmodium falciparum var* gene regulation"
5/2011	Oral presentation, 7th Annual BioMalPar Conference, Heidelberg, Germany, „Mutually exclusive activation of *Plasmodium falciparum var* genes is mediated by interplay of autonomous regulatory elements"
9/2010	Poster presentation, 21st Molecular Parasitology Meeting, Woods Hole, MA, USA
5/2008	Poster presentation, 4th Annual BioMalPar Conference, Heidelberg, Germany

Publications

Brancucci NMB, Witmer K, Schmid CD, Flueck C, Voss TS. Cell Microbiol. 2012 Dec; 14(12):1836-48. Identification of a cis-acting DNA-protein interaction implicated in singular *var* gene choice in *Plasmodium falciparum*.

Oehring SC, Woodcroft BJ, Moes S, Wetzel J, Dietz O, Pulfer A, Dekiwadia C, Maeser P, Flueck C, Witmer K, **Brancucci NMB**, Niederwieser I, Jenoe P, Ralph SA, Voss TS. Genome Biol. 2012 Nov 26; 13(11):R108. Organellar proteomics reveals hundreds of novel nuclear proteins in the malaria parasite *Plasmodium falciparum*.

Witmer K, Schmid CD, **Brancucci NMB**, Luah YH, Preiser PR, Bozdech Z, Voss TS. Mol Microbiol. 2012 Apr;84(2):243-59. Analysis of subtelomeric virulence gene families in *Plasmodium falciparum* by comparative transcriptional profiling.

Honours

9/2012	Poster Prize at the 23rd Molecular Parasitology Meeting, Woods Hole, MA, USA „Functional Investigation of *Plasmodium falciparum* Heterochromatin Protein 1"
5/2012	Poster Prize at the 8th Annual BioMalPar Conference, Heidelberg, Germany „Exclude and Repress – A dual role for 5` upstream regions in *Plasmodium falciparum var* gene regulation"
9/2010	Poster Prize at the 21st Molecular Parasitology Meeting, Woods Hole, MA, USA „The role of regulatory promoter elements in epigenetic regulation of *P. falciparum var* genes"
3/2004	„Basler Maturandenpreis der Novartis" for the best degree in 2003 at the academic high school of Economics and Law „Wirtschaftsgymnasium" in Basel, Switzerland

Hobbies

Rock climbing, Squash, Volleyball, Intercrosse

British classic cars

Languages

German:	Native tongue
English:	Fluent in speaking and writing
French:	Intermediate in speaking and writing

References

Till S. Voss Swiss
Tropical and Public Health Institute
Socinstrasse 57
4051 Basel
till.voss@unibas.ch

Hans-Peter Beck
Tropical and Public Health Institute
Socinstrasse 57
4051 Basel
hans-peter.beck@unibas.ch

During my studies, I attended lectures of the following lecturers:

H. Walser, H. Wennemers, C. Körner, D. Senn, J. Stöcklin, J. Oetiker, P. Oehlhafen, B. Hecht, A. Rolink, K. Beier, H. Hirsch, T. Klimkait, W. Meier, M. Nyffeler, D. Ebert, H.P. Hauri, M. Hall, T. Bickle, U. Jenal, W. Gehring, S. Zschokke, W. Keller, M. Affolter, G. Cornelis, C. Dehio, U. Aebi, H. Reichert, T. Boller, B. Baur, A. Wiemken, R. Brun, N. Weiss, M. Tanner, H.P. Beck, I. Felger, G. Pluschke, P. Vounatsou