

Review Article

Telomeric Heterochromatin in *Plasmodium falciparum*

Rosaura Hernandez-Rivas,¹ Karla Pérez-Toledo,^{1,2} Abril-Marcela Herrera Solorio,¹
Dulce María Delgadillo,¹ and Miguel Vargas¹

¹Departamento de Biomedicina Molecular, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (IPN), Apartado Postal 14-740, 07360 México, DF, Mexico

²Departamento de Genética y Biología Molecular, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (IPN), Apartado Postal 14-740, 07360 México, DF, Mexico

Correspondence should be addressed to Rosaura Hernandez-Rivas, rohernan@cinvestav.mx

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Until very recently, little was known about the chromatin structure of the telomeres and subtelomeric regions in *Plasmodium falciparum*. In yeast and *Drosophila melanogaster*, chromatin structure has long been known to be an important aspect in the regulation and functioning of these regions. Telomeres and subtelomeric regions are enriched in epigenetic marks that are specific to heterochromatin, such as methylation of lysine 9 of histone H3 and lysine 20 of histone H4. In *P. falciparum*, histone modifications and the presence of both the heterochromatin “writing” (PfSir2, PKMT) and “reading” (PfHP1) machinery at telomeric and subtelomeric regions indicate that these regions are likely to have heterochromatic structure that is epigenetically regulated. This structure may be important for telomere functions such as the silencing of the *var* gene family implicated in the cytoadherence and antigenic variation of these parasites.

1. Introduction

Plasmodium falciparum, a protozoan that is the causal agent of the most severe form of human malaria, has a complex life cycle with two different hosts: the *Anopheles* mosquito and humans. In order to complete its life cycle, *P. falciparum* invades different types of cells and self-propagates in very distinct environments in the mosquito (gut, hemolymph and salivary glands) as well as in the human host (skin, liver and erythrocytes). Each of these distinct environments exerts selective pressures related to morphological changes and forces *P. falciparum* to exhibit differential gene expression during its life cycle [1–3]. Transcriptome analyses have shown that the parasite displays tightly coordinated cascades of gene expression [2, 3]. However, the molecular mechanisms that drive this regulation have not yet been deciphered. Surprisingly, the *P. falciparum* genome encodes a relatively low number of transcription factors, although the basal core transcriptional machinery and protein-coding genes involved in nucleosome assembly and the regulation of chromatin structure are conserved [4–6]. To date, no

specific DNA-binding proteins have been identified other than PfTBP [7], PfMyb1 [8] and members of the ApiAp2 gene family [9]. These observations suggest that epigenetic mechanisms play a significant role in the control of gene expression in *P. falciparum* [4, 6, 10]. We define “epigenetic” as the situation in which the activation and silencing of individual genes are inherited in the absence of changes in the DNA sequence. With this definition in mind, we will begin by providing a brief overview of chromatin and telomere structure, followed by a summary of the proteins and epigenetic modifications involved in heterochromatin assembly in this parasite. Finally, we discuss the potential role of heterochromatin in the control of the *var* genes expression.

2. *P. falciparum* Chromosome Organization

The genome of *P. falciparum* is 22.8 Mb in total, organized in 14 chromosomes whose sizes range from 0.7 to 3.4 Mb [11]. Chromosome mapping has revealed that the chromosomes are compartmentalized, containing conserved regions at

their central domains and polymorphic regions at their terminal domains. Thus, the housekeeping genes are located within the central regions of the chromosomes, whereas the highly variable gene families responsible for the antigenic variation of the parasite are clustered towards the telomeres [12]. The chromosome ends are made up of telomeric repeats (GGGTTT/CA) organized in tandem, followed by an array of noncoding DNA elements at the subtelomeric regions (Figure 1(a)). These telomere-associated sequences (TAS) are species-specific and consist of a coding and a noncoding region. The non-coding region is composed of a mosaic of six different blocks of repetitive sequences located between the telomere and the coding regions. These elements are called “telomere associated repetitive elements” (TAREs) and span 20–40 kb (Figure 1(a)) [13, 14]. The six elements are positioned in the same relative order on all the chromosomes, while the size and the DNA sequence of each TARE are polymorphic [11, 13]. Adjacent to the non coding TAREs are members of multi-gene families that are involved in immune evasion and cytoadhesion (*var* genes) and putative variant antigens (*rif* and *stevor* genes) [11, 13].

3. Chromatin in *P. falciparum*

As in all eukaryotic organisms, chromatin in *Plasmodium* is organized into fundamental units called nucleosomes [15]. These units restrict the binding of transcription factors to their target sequences and regulate gene expression. *P. falciparum* contains genes coding for each of the four histones required for the assembly of nucleosomes: H2A, H2B, H3 and H4, as well as the histone variants H3.3, CenH3, H2AZ and H2Bv [16]. However, no gene encoding for histone H1 has been identified in this parasite to date, which is particularly significant since histone H1 is responsible for binding the DNA linker present between two nucleosomes and helping to compact the chromatin into the canonical 30 nm fiber.

It is feasible that chromatin structure might contribute to the regulation of gene expression in *P. falciparum* because the histones that make up the nucleosomes in their chromosomes have a primary structure very similar to those found in other eukaryotes.

In most organisms, histones H3 and H4 are among the most conserved proteins. Their amino terminal ends, which are necessary for the formation of the nucleosomal core, are fairly conserved in terms of their sequence, particularly at residues that are susceptible to specific covalent modifications [17]. The amino terminal ends of histones H3 and H4 of *P. falciparum* contain sites susceptible to post-translational changes (Figure 1(b)). Several modifications have been identified in *P. falciparum* by mass spectrometry analysis of histones, Western blot assays performed with antibodies specific to methylated and acetylated histones, and ChIP on ChIP assays. Those modifications are: acetylation of histone H3 at residues K9, K14, K18, and K27 [16, 18–20] and of histone H4 at K5, K8, K12 and K16 [16, 21]; methylation of histone H3 at K4, K9 and K36 [20, 22, 23] and histone H4 at K20, as well as the sumoylation of histone H4 [24]. Recently, a comprehensive mass spectrometry analysis of *P.*

falciparum histones identified 44 new post-transcriptional modification sites in these proteins, most of them associated with a transcriptionally permissive state (Figure 1(b)) [25].

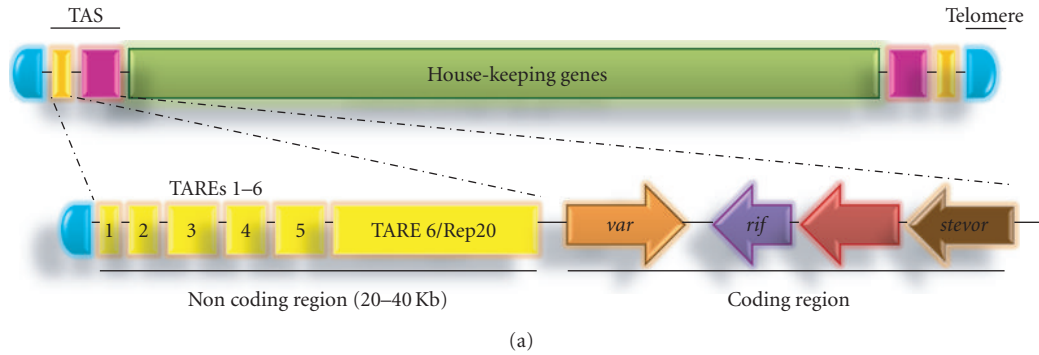
Additionally, both biochemical and molecular studies have demonstrated that this parasite contains chromatin-modifying enzymes, including histone deacetylases (PfHDAC1-Rpn3, PfHDAC2 and PfSIR2) and the PfGcn5 histone acetyl transferase (HAT) [26]. Bioinformatic analyses conducted in the last year have identified at least nine genes encoding histone lysine methyl transferases (HKMTs) in the *P. falciparum* genome (PfHKMT1–9) [22]. One methylase that belongs to the SET 8 family, displays H4K20 mono-, di-, and trimethylase activities [27]), and another HKMT (SET 3) is likely involved in the methylation of histone H3K9 [22]. The same study showed that the genome of *P. falciparum* also encodes several proteins that contain the JmjC domain, which removes methyl groups from histones (histone demethylases) [22]. However, SNFL2 is the only *P. falciparum* ATP-dependent chromatin-remodeling enzyme that has been characterized [28].

Although several proteins responsible for introducing histone modifications (writers) have been identified in *Plasmodium*, none of the proteins that recognize the modifications (readers) had been identified in the parasite until recently; the HP1 protein of *P. falciparum* (PfHP1) was identified [29]. PfHP1 contains domains that are characteristic of HP1 proteins, such as a chromo-domain (CD) and a chromo-shadow domain (CSD) [29]. As in higher eukaryotes, PfHP1 recognizes dimethylated or trimethylated H3K9 through its CD, and it can form homodimers, probably through its CSD [29]. These data suggest that this protein has an important role in the compaction of nucleosomes, activity required to form heterochromatin.

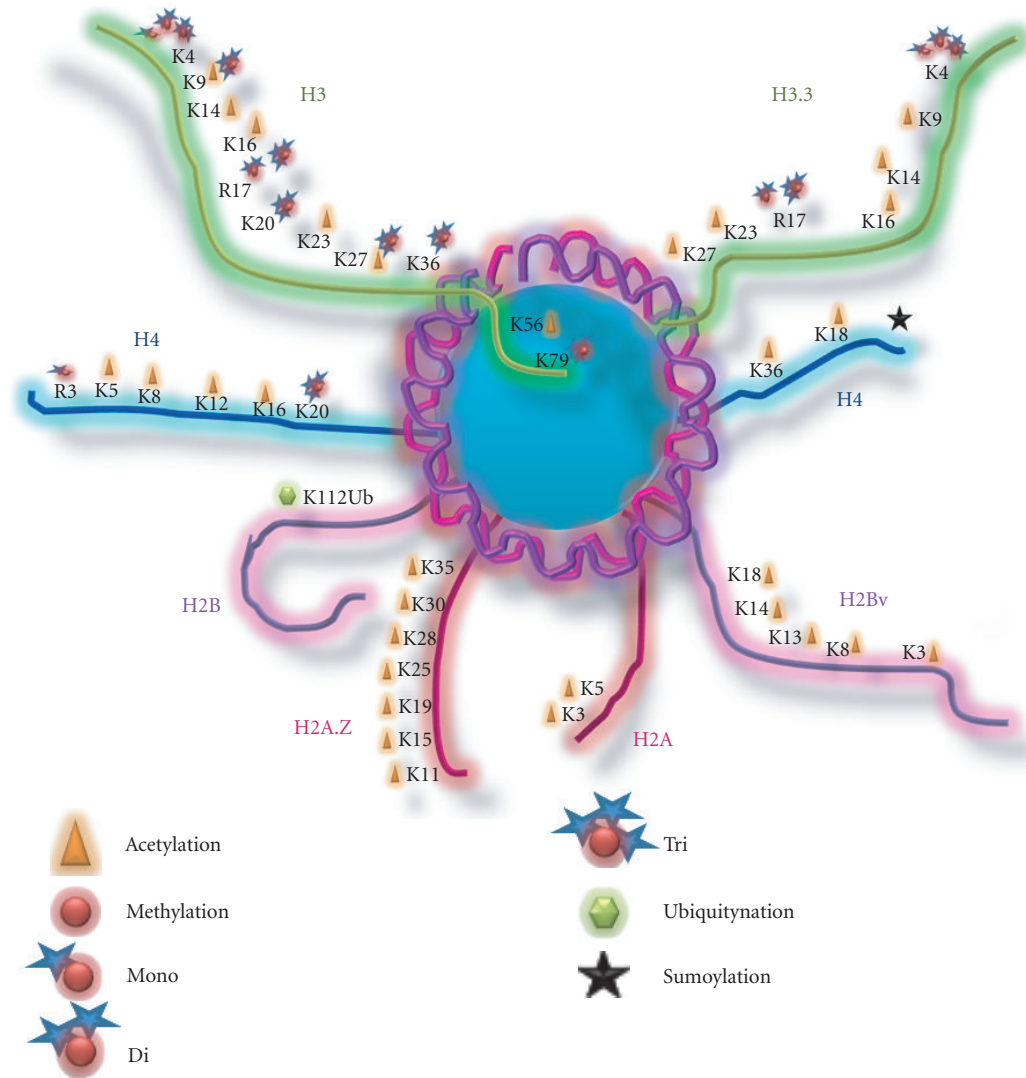
4. The Function of Post-Translational Modifications in Histones

The various chemical modifications that occur at the amino terminal of the histones affect chromatin architecture, that is, they help establish the functional and structural domains known as euchromatin and heterochromatin. Euchromatin, is open chromatin that allows transcription factors to access and transcriptionally activate their target genes. Euchromatin, which is maintained largely by housekeeping genes, is condensed during metaphase and decondensed during interphase. Heterochromatin differs from euchromatin in that it is condensed during interphase. It is often said to be “poor in genes” and mainly constituted for repetitive DNA sequences. Moreover, since it is highly condensed and inaccessible to transcription factors, heterochromatin is generally transcriptionally silent [30].

The formation and maintenance of heterochromatin depends on chromosomal localization, nuclear localization, and the presence and density of repeated DNA elements [31]. Chromosomal regions that contain a high density of repeated DNA elements organized in clusters, such as telomeres and centromeres, are the primary targets for the formation of heterochromatin. These regions remain condensed throughout the cell cycle and are known as “constitutive



(a)



(b)

FIGURE 1: A model of *P. falciparum* chromosome organization. (a) The *P. falciparum* haploid nuclear genome is organized into 14 linear chromosomes. Each chromosome is composed of an internal region in which housekeeping genes are located, and a chromosome end that displays a higher-order DNA organization common to all *P. falciparum* chromosomes. Downstream of telomeres resides at highly polymorphic TAS composed of a noncoding and a coding region. The noncoding region contains six TAREs, which are always positioned in the same order but are of variable length. TARE6, also known as Rep20, is responsible for most of the length polymorphism observed in the noncoding region of the TAS. The coding region contains several gene families encoding important virulence factors, such as the *var* and *rifin* genes. (b) A schematic representation of post-translational histone modifications in *P. falciparum*. The amino acids on the N-terminal tails of histone H2A, H2B, H3, H4 and the histone variants H2A.Z and H3.3 that are subject to different modifications are described in the figure.

heterochromatin". However, heterochromatin is also found at loci that are regulated during development; the chromatin state switches from compact (heterochromatin) to open (euchromatin) in response to cellular signals that regulate gene activity (e.g., inactivation of the X chromosome and the senescence-associated heterochromatin SAHF locus). This is known as "facultative heterochromatin" [32]. Facultative heterochromatin is interspersed with euchromatin and is organized in small domains along the chromosomes [30, 33–35]. The domains may contain promoter regions of individual genes or chromosomal domains that need to be silenced [36]. Facultative heterochromatin may be specific of cell type and/or of cell differentiation stage, and even more, appear just in one of two homologous chromosomes [30, 34, 35].

5. Post-Translational Histone Modifications and Their Role in Telomeric Heterochromatin Formation

Studies from yeast to mammals strongly suggest that histones and their modifications have an important role in the assembly of heterochromatin. The increase in the acetylation or methylation of specific histone residues (e.g., H3K9ac, H3K4me and H3K36me) invariably correlates with transcriptional activity; a decrease in acetylation levels is correlated with a repressed transcriptional state. Therefore, heterochromatin is associated with histone hypoacetylation [37]. Furthermore, the methylation of H3K9 or H4K20 and the sumoylation of the four histones are characteristic heterochromatin markers [38]. In general, the methylation of specific lysine residues in the amino terminal ends of the core histones is critical for the establishment, maintenance and silencing of chromatin domains in the chromosome centromeric and telomeric regions.

6. Heterochromatin Assembly Is an Orchestrated Mechanism

So far, we have described how epigenetic marks such as H3K9me3 and histone deacetylation are associated with transcriptional repression and occur frequently in histones at the telomeric and subtelomeric regions of linear chromosomes. We have also discussed how proteins such as histone methyltransferases and histone deacetylases are involved in the formation of a hypoacetylated state that correlates with increased levels of methylation, which in turn leads to the formation of a more compact chromatin structure. Finally, we have seen that in addition to these proteins that alter the amino ends of the histones, enzymes like protein PfHP1 are required to read these histone modifications and translate this information into biological processes in the cell. Thus, the recognition of histone methylation by PfHP1 and the resulting chromatin compaction leads to the silencing of gene expression. However, in order to ensure that all this machinery is in the right place, the appropriate region or regions for heterochromatin formation must be recognized in a sequence-specific manner. The current understanding in

the mechanisms to produce this telomeric heterochromatin will be described in the following sections.

7. Composition and Chromatin Structure of Telomeric Regions

Telomeres are DNA-protein complexes that stabilize the ends of linear chromosomes in eukaryotes [39]. Telomeres perform several essential functions, allowing the complete replication of chromosomes, providing a cap that protects chromosome ends from degradation by nucleases and preventing chromosome fusion. The absence of telomeres results in genetic instability and loss of cellular viability [39, 40]. The subtelomeric regions located next to telomeres are composed of repetitive DNA and contain few genes. The telomeric DNA consists of tandem short repeats of G-rich sequences (TTGGGG for *Tetrahymena* and TTAGGG in humans) [41]. The telomeres of *P. falciparum*, present at the ends of its 14 linear chromosomes, consist of the sequence GGGTT(T/C)A, which is highly conserved among the different species of *Plasmodium* [42]. The average telomere length varies among different species of the parasite. For example, it has been shown that the telomere regions of *P. falciparum* are 1.2 kb long, while those in *P. vivax* are 6.7 kb long. The relatively conserved telomere length among the different strains of *P. falciparum* can be considered specific to the species [42].

Differences in telomere length may be due to differences in the organization of chromatin in these regions. Micrococcal nuclease assays have established that the innermost part of telomeres in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have a nucleosomal organization that consists of three or four nucleosomes (Figure 2). The outermost part of the telomeres, known as the telosome, is not associated with nucleosomes [43]. Instead, this portion of the chromosome is bound by a large number of telomere-specific proteins that form the telosome complex. In *S. cerevisiae*, for example, multiprotein complexes such as Rap1, the Ku complex, Taz1, Rif 1, the Mlp complex, telomerase and the SIR silencing complex comprised of the proteins Sir2, Sir3 and Sir4 [44, 45] have been isolated and characterized. Telomeres in *P. falciparum* have an organization similar to that of the telomeres in *S. cerevisiae*, with an internal region associated with three to four nucleosomes and an outer region free of them [13]. Several proteins orthologous to those in the yeast telosome complexes, such as PfSir2 [21], PfOrc1 [46] and telomerase (PfTERT) [47], have also been found in this parasite. All these findings support the existence of a telosome complex in *P. falciparum* (Figure 2).

8. The Telosome Complex and Its Participation in the Assembly of Telomeric Heterochromatin

The telosome complex performs multiple functions, such as the protection of the terminal ends of linear chromosomes from degradation by exonucleases and prevention of recombination of heterologous telomeres. Telosomes also

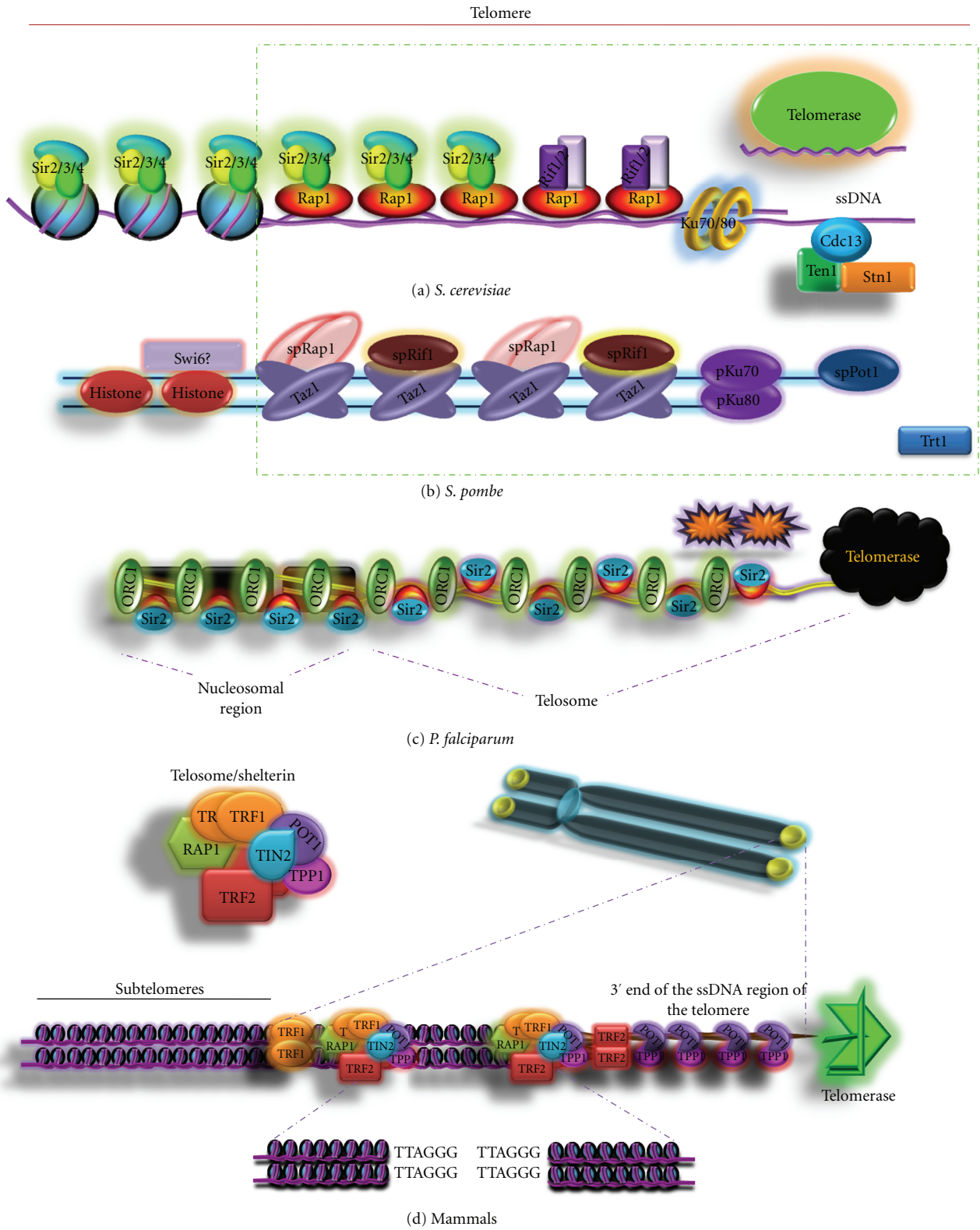


FIGURE 2: Heterochromatin in *S. cerevisiae*, *S. pombe*, *P. falciparum* and human.

participate in the replication of telomeric regions, help to anchor telomeres to the nuclear periphery, control the length of telomeres, influence the formation of telomeric clusters and heterochromatin, and affect the regulation of the expression of genes adjacent to subtelomeric and telomeric regions through a telomere position effect (TPE) [14, 44, 45, 48].

But how does the telosome complex participate in the formation of telomeric heterochromatin? In *S. cerevisiae*, one of the proteins that bind to the telosome is Rap1, which recognizes telomeric repeats through its two Myb domains [49]. Once Rap1 binds to telomeric DNA in a sequence-specific manner, it can recruit Sir2, Sir3, and Sir4 [50]. Once this silencing complex has been formed, the full SIR complex is able to spread along the adjacent sequences because of the high affinity that Sir3 and Sir4 have for hypoacetylated histones H3 and H4, thus generating heterochromatin structure (Figure 2(a)) [51–54]. The spreading of the SIR complex may require the active deacetylation of histone tails next to the SIR complex performed by Sir2, as spreading appears to be counteracted by the histone acetylase Sas2 [55].

The heterochromatin assembly machinery in *S. pombe* is more similar to that of mammals than that of *S. cerevisiae*. The assembly process begins when Taz1 protein binds to telomeric repeats and recruits the proteins spRap1 and spRif1. *S. pombe* does not have Sir3 and Sir4 orthologues, but rather the HP1 orthologue Swi6. This protein contains chromo and chromo-shadow domains, which are involved in the formation of protein complexes and association with chromatin. As in higher eukaryotes, the assembly of heterochromatin requires the action of a histone methyltransferase (called Clr4 in *S. pombe*), which methylates H3K9 to create a binding site for Swi6. In addition, the deacetylation of H3K14 seems to be important for the silencing and recruitment of Swi6 to heterochromatic regions. Once Swi6/HP1 has bound the chromatin, the methylation of histones continues, thus creating additional binding sites for Swi6 on adjacent nucleosomes. This allows the histone modifying enzymes and Swi6 to extend in *cis* beyond the site where the heterochromatin complex was nucleated (Figure 2(b)) [39].

The repeats present in the subtelomeric regions also contribute to the formation of telomeric heterochromatin through the formation of the RIST complex (RNA-induced initiation of transcriptional gene silencing). This transcriptional silencing machinery uses small non-coding RNAs (siRNA) to signal from regions where H3K9 is methylated as well as to assemble heterochromatin on the repetitive DNA [32]. The current model proposes that the methylation of H3K9, established by the RNA interference (RNAi) machinery in *trans* and/or by DNA binding factors, anchors the RIST complex to chromatin in a stable fashion. This enhances the ability of the RDRC complex (RNA-dependent RNA polymerase complex) and Dicer enzyme to process siRNA from nascent transcripts. The generated siRNA is responsible for directing histone-modifying enzymes such as the Clr4 methyltransferase toward the repetitive elements of DNA in order to methylate them. Then, the H3K9me3 epigenetic mark recruits chromo-domain-containing proteins such as

Chp1, Chp2 and Swi6. Chp1 is part of the RITS complex, so this recruitment establishes a new siRNA transcription process that will promote the recruitment of Clr4 in order to establish the structure of heterochromatin more efficiently [32].

Similar to yeast, in mammals a multiprotein complex known as shelterin binds to the telomeric repeats. The shelterin complex contains orthologues of the yeast proteins that bind both to the repeats present in double-stranded DNA and to a broken G end. The assembly of this heterochromatin begins when TRF1 and TRF2 bind to telomeric repeats. In this way, TRF1 interacts with TIN2 as well as with the proteins TANK1 and TANK2. It has been suggested that TRF1, and other proteins that interact with it, act as negative regulators of telomere length. On the other hand, TRF2 and POT1 appear to have additional functions in the protection of the telomeres, particularly in preventing the fusion of chromosome ends. Sirt1 and SUV39 (HMT) are enzymes responsible for the modulation of chromatin that accompanies the formation of facultative heterochromatin. SUV39H1 forms histone H3 that contains a trimethyl group at lysine 9 (H3K9me3) in heterochromatic regions. Sirt1 is a NAD-dependent deacetylase that removes acetyl groups from H4K16. Moreover, Sirt1 directly interacts with SUV39H1 and deacetylates HMT, which in turn increases the activity of HMT and thus H3K9me3 levels. Even when H3K9 has been methylated by the action of the histone methyltransferase, this change alone is not enough to establish highly condensed chromatin. In order to achieve this effect, it is necessary to recruit the non-histone HP1 protein [56]. HP1 binds to histone H3 when it recognizes di- or trimethylated lysine 9 through its chromo-domain, so HP1 colocalizes with Su(var)3–9 in the heterochromatin regions (Figure 2(d)) [39].

One question that arises is how HP1 contributes to the generation of highly condensed chromatin and the repression of gene expression? This mechanism is not yet fully understood, but it has been suggested that the recruitment of HMT can lead to H3K9 trimethylation, which in turn is recognized by the HP1 CD. The recruitment of HP1 through H3K9me3 could promote HP1 dimerization via its CSD, which would allow HP1 to spread over the subtelomeric region, resulting in a more compact chromatin structure [34]. It has been demonstrated recently that HP1 is not only associated with constitutive heterochromatin, but is also involved in the silencing of specific genes within euchromatin to form facultative heterochromatin [34, 57, 58].

9. Telomeric and Subtelomeric Assembly of Heterochromatin in *P. falciparum*

P. falciparum possesses short chromosomes, and until very recently, heterochromatin was not observed in this parasite. The repetitive structure of the subtelomeric DNA [13] and the association of telomeres in *clusters* at the nuclear periphery [59] suggested the existence of heterochromatin at the ends of the chromosomes. Moreover, chromosome-painting studies revealed that a pair of subtelomeric probes

separated by 60 to 80 kb colocalized at the same spot in the nucleus, while two similarly distant internal probes are seen as two distinct signals [21]. These data indicated that the regions proximal to the telomeres (known as telomere-associated repeat elements; TAREs, and coding region that containing the *var*, *rifin* and *stevor* genes) exist in a more condensed form than the inner regions of the chromosome. Consistent with this, electron-dense heterochromatin was observed at the periphery of *P. falciparum* nuclei in ultrathin sections [21]. All of these data support the existence of telomeric and subtelomeric heterochromatin in *P. falciparum*. Furthermore, ChIP assays demonstrated that in *Plasmodium*, the histone deacetylase PfSir2 can associate indirectly with telomeres and with all the subtelomeric repetitive elements until TARE6, also known as Rep20. Therefore, it is proposed that this protein may generate a heterochromatin gradient from telomeric regions to TARE6 [59].

Heterochromatin formation in *S. cerevisiae* depends only on the SIR silencing complex formed by Sir2, Sir3 and Sir4. Sir2 is the only member of this complex that has been identified and characterized in *P. falciparum* [21, 60]. A recent search for genes encoding Sir3 and Sir4 orthologues in *P. falciparum* had not succeed; instead, Orc1 (PfOrc1), whose amino end is highly similar to that of the Sir3 protein, was identified [46]. It has been demonstrated that the amino end of Orc1 is involved in the formation of heterochromatin at the *S. cerevisiae* mating locus [61], and that Orc1 is also part of the protein complex that participates in the assembly of telomeric heterochromatin in *Drosophila* and *Xenopus* [62–64]. All these data suggest that this protein is involved in the assembly of heterochromatin. Furthermore, immunofluorescence assays showed that PfOrc1 localizes in telomeric clusters. We have used EMSA and ChIP assays to demonstrate that PfORC1 can specifically interact with telomeres and subtelomeric elements similar to PfSir2, making PfORC1 a strong candidate for contributing to telomeric silencing in *P. falciparum* [46]. Another finding was that PfORC1 associates with TARE6 and that this association is not sequence-dependent but rather structure-dependent [46]. Our results lead us to speculate that *P. falciparum* chromosome ends may fold back on themselves, allowing telomeric chromatin to interact with subtelomeric heterochromatin. This represents the first evidence of a structure similar to a t-loop in *P. falciparum*, which may account for the stabilization of telomeric and subtelomeric chromatin at the nuclear periphery, as has been suggested for yeast. Interestingly, the PfORC1-DNA interaction is mediated by a putative leucine zipper DNA-binding motif present in the N-terminal region of the protein [46, 65]. We hypothesize that similar to Rap1 in yeast; ORC1 could recruit PfSir2 protein to the chromatin, since PfSir2 has no evident DNA-binding motifs. Although no interaction between PfORC1 and PfSir2 was observed in a yeast two-hybrid assays [66], PfORC1 may act as the initiator of heterochromatin assembly and be involved in the indirect recruitment of PfSir2 to the telomeric repeats [46]. Besides PfSir2 and PfOrc1, the nonhistone protein HP1 is also thought to be involved in heterochromatin formation in *P. falciparum*. Recently, also was demonstrated by ChIP assays

that PfHP1 protein binds strongly to the whole subtelomeric region [29]. Therefore, this protein together with PfSir2 and PfOrc1 may participate in the formation and establishment of telomeric and subtelomeric heterochromatin.

In order to explain the formation and assembly of heterochromatin in *P. falciparum*, a model that considers these data (analogous to the formation of telomeric heterochromatin in *Drosophila*) has been suggested. Initially, PfORC1, which possesses a DNA binding domain, could directly recognize the telomeric/subtelomeric repeats, as occurs at the HM loci in yeast, resulting in PfHP1 recruitment. ORC has been shown to be necessary for HP1 targeting to chromatin [67]. In parallel, another as yet unknown factor would recruit PfSir2 to the telomeric repeats, deacetylating H3K9 along the subtelomeric region. Once this is done, this residue would be accessible for methylation, probably by PfSet3 (a histone lysine methyltransferase). The recruitment of a histone lysine methyltransferase would lead to H3K9 trimethylation, which in turn is recognized by the chromodomain of PfHP1. PfHP1 recruitment through H3K9me3 could promote PfHP1 dimerization via its CSD, which would allow sequential PfHP1 spreading over the subtelomeric region, resulting in a more compact chromatin structure [29] (Figures 2(c) and 3).

10. The Var Genes as an Example of Transcriptional Silencing by TPE in *P. Falciparum*

Telomeric chromatin has several characteristics similar to those of centromeric heterochromatin for example, the silencing of nearby genes. The silencing of genes located near centromeres is known as a variegation effect (VE), while silencing of genes near the telomeres is known as TPE [52]. TPE is caused by the spread of heterochromatin in a Sir protein-dependent manner. Since its original characterization in yeast, numerous studies in various organisms have reported several protein modulators and effectors of TPE, as well as nuclear and chromosomal contexts that are required for this effect to take place [68].

The first evidence of transcriptional repression mediated by telomeres in *P. falciparum* arose from a study that compared the transcription of the gene HRP1 located 100 kb from the telomeric repeats on chromosome 2 with the transcription of the same gene relocated next to the telomere [69]. Quantitative RT-PCR analysis showed that the transcription of the HRP1 gene located next to the telomere was 50 times lower than the transcription of the same gene at an internal locus. These results suggested that HRP1 was silenced by the heterochromatin that had spread from the telomere to proximal regions. Therefore, it was proposed that telomeres alone are capable of producing a partial TPE effect for this gene [46].

To date, the *var* genes, which encode the PfEMP1 protein that plays essential roles in cytoadherence and antigenic variation in *P. falciparum*, are the only genes in this organism known to be regulated by chromatin remodeling. PfEMP1 proteins are encoded by a multigene family that consists

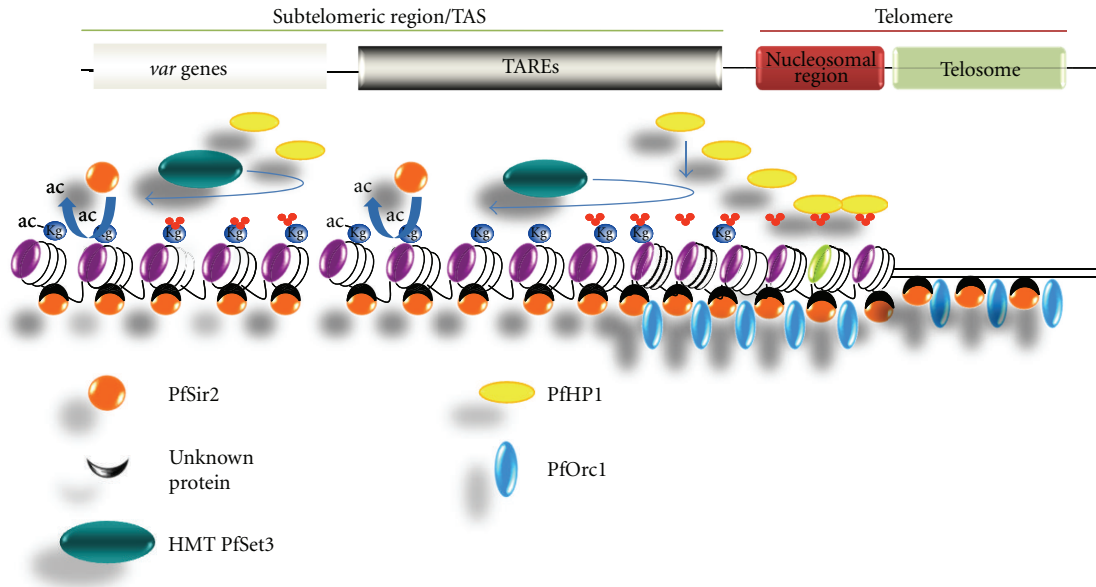


FIGURE 3: *Hypothetical model for heterochromatin assembly at P. falciparum chromosome ends.* This is a general view of the known chromatin components at *P. falciparum* subtelomeres. The spreading of heterochromatin along the different TAREs into adjacent coding regions probably involves PfHP1, PfSir2 and PfKMT1 in cooperation. The role of PfOrc1 in this process remains unknown.

of 60 *var* genes [70]. The *var* genes are regulated at the transcriptional level in such a way that only one *var* gene is transcribed at any given moment while the remaining 59 are transcriptionally silent [71]. Thus, the parasite must have some regulatory mechanisms that coordinate the expression of this gene family to ensure that only one gene is expressed in each organism. Evidence that the regulation of *var* genes occurs at the epigenetic level emerged from studies performed by Deistch KW and Voss T., who found that when the promoter region of a *var* gene (type Ups C or Ups B) was transfected transiently, it was then expressed in a constitutive rather than a stage-specific manner [72, 73]. This suggested that the chromosomal context of the *var* promoter is crucial for the silencing process, and that chromatin might play an important role in the regulation of the expression of this multigene family. The epigenetic control of *var* genes was clearly demonstrated in 2005 by Scherf and colleagues who showed that the PfSir2 protein is present at the perinuclear heterochromatin and telomeres. Moreover, super-shift and chromatin immunoprecipitation (ChIP) assays with the PfSir2 antibody showed that this protein is able to immunoprecipitate telomeres and TARE6 elements [21]. Later, using the same antibody (anti-PfSir 2), this protein was found associated with telomeric regions as well as the TARE1, 2, 3, and 6 elements, suggesting that PfSir 2 is capable of generating a heterochromatin gradient from the telomeres to TARE6, covering a distance of 50 kb. [46]. Some *var* genes are located adjacent to TAREs, which suggests the hypothesis that PfSir2 spreads towards the *var* loci and inhibits the expression of these subtelomeric *var* genes. Again, ChIP assays using the anti-PfSir2 antibody and two populations of parasites, each of which expressed a different *var* gene (FCR3-CSA, in which only the *var2CSA*

gene is active, and FCR3-CD36, in which only *var*-CD36 is transcribed), demonstrated that PfSir2 is associated with the Ups E promoter (which directs the gene expression of *var2CSA*) when it is transcriptionally inactive, but not when it is active. This experiment showed that PfSir2 is involved in the repression of the subtelomeric *var2CSA* gene through the extension of subtelomeric heterochromatin toward the gene in a mechanism similar to TPE. In addition, ChIP studies using antibodies against acetylated histone H4 demonstrated that the promoter of *var2CSA* associates with acetylated H4 only when it is transcriptionally active and not when it is repressed. All of these results suggest that the transcriptional activity of the subtelomeric gene *var2CSA* is reversibly regulated by epigenetic mechanisms through the acetylation and deacetylation of histones [21] (Figure 3).

These data were later confirmed with quantitative ChIP assays in the same parasite populations (FCR3-CSA, *var2CSA*-on; FCR3-CD36, *var2CSA*-off). The results of these experiments established that tri- and dimethylated H3K4 and acetylated H3K9 are enriched in the 5' UTR region of the active *var2CSA* gene during the ring stage; whereas H3K4me2 is highly represented at this locus during the trophozoite and schizont stages, when *var2CSA* is not actively transcribed. Thus, these modifications have proven to be a signal that allows the parasite to "know" which one of the *var* gene is going to be reactivated in the next intraerythrocytic cycle (poised state) [20]. The results also suggest that H3K4me2 may be contributing to the establishment of cell memory, thus helping to maintain the same pattern of monoallelic expression for several generations. Moreover, the same study showed that when the gene *var2CSA* is transcriptionally inactive, H3K9me3 is highly represented both in the 5' UTR region of this gene and throughout

its coding region. Therefore, the trimethylation of H3K9 reflects an inactive or repressed state of the *var2CSA* gene and acts antagonistically towards H3K9ac and H3K4me3 [20].

A complementary microarray study performed by Cowman's team, comparing global transcription in wild-type versus Δ Sir2 parasites, showed a significant increase in transcripts for a subset of *var* (*upsA* and *upsE*) and *rifin* genes. However, the *var* genes found at internal chromosomal loci, were similarly repressed in wild-type and Δ Sir2, which indicates that PfSir2 is not required to silence these *var* genes. This result is consistent with PfSir2 ChIP experiments performed in our lab in which PfSir2 was not found at the promoters of internal *var* genes. These data suggest that different mechanisms mediate the silencing of subtelomeric and central *var* genes [21, 74].

Recently, ChIP on ChIP assays had shown that H3K9me3 is highly enriched at subtelomeric regions associated with TAREs, subtelomeric *var* genes, *rifins* and *stevor* genes at the ends of all chromosomes, as well as in the central region of chromosomes 4, 6, 7, 8 and 12 where the central *var* genes are located. This mark is strongly associated with *var* genes that are transcriptionally inactive. These data reinforce the idea that the H3K9 trimethylation plays an important role in the transcriptional silencing of both the subtelomeric and the central *var* gene. Therefore, H3K9me3 is an epigenetic mark that seems to regulate the silencing of the *var* genes in *P. falciparum* [20].

11. Conclusions and Perspectives

The study of constitutive heterochromatin has gained interest as significant experimental evidence shows that this type of heterochromatin, which was long considered transcriptionally inactive, is sometimes transcribed in *S. pombe*, mice and humans [39, 75]. Moreover, non-coding RNAs appear to play a very important role in the assembly and formation of heterochromatin in these three organisms. On the other hand, the clear participation of telomeric heterochromatin in the regulation of gene expression in parasites, fungi and humans (as well as in the transcriptional activity in these regions) demands a review of the concept of facultative heterochromatin, which has been given only a structural role.

The initial evidence that constitutive heterochromatin might be transcribed in *P. falciparum* was provided when Deitsch and colleagues identified the first transcripts from the centromeric repeats of the parasite; this group also showed that non-coding RNA is associated with centromeric regions [76]. Meanwhile, no transcriptional activity has been reported in the telomeric or subtelomeric regions of this protozoan so far, despite the important role that these regions have in the silencing of subtelomeric type A and type E *var* genes. Telomeric heterochromatin could also participate in regulating the expression of other genes involved in clonal variation, such as *rifins* and *stevor*, all of which are located in subtelomeric regions. Therefore, in order to understand what controls the expression of the proteins most directly involved in the virulence of this parasite, it is of great importance to establish which ones are

the proteins and/or other elements involved in the assembly of heterochromatin in *P. falciparum*.

A better characterization of the heterochromatin and euchromatin in *P. falciparum* will allow us to further elucidate the regulation of the complex process of gene expression dynamics. Combining of ChIP technology with microarray analysis may give us the tools to understand how epigenetic factors are linked to different aspects of the parasite's chromosome biology, as well as generate potential avenues for malaria intervention strategies.

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