Transcriptional variation in malaria parasites: why and how

Oriol Llorà-Batlle[1,#], Elisabet Tintó-Font[1,#] & Alfred Cortés[1,2,*]

[1] ISGlobal, Hospital Clínic - Universitat de Barcelona, 08036 Barcelona, Catalonia, Spain

[2] ICREA, 08010 Barcelona, Catalonia, Spain

# These authors contributed equally to this work and are listed in alphabetical order.

*Corresponding author.

Telephone: +34 93 2275400; E-mail: alfred.cortes@isglobal.org

BIOGRAPHIES

Alfred Cortés is an ICREA Research Professor at ISGlobal (Barcelona). He has previously worked in diverse aspects of malaria research in PNG and Europe. His main current research interests are malaria epigenetics and sexual conversion.

Oriol Llorà-Batlle is a PhD Student at ISGlobal. He pursued a BSc in Biology and a MSc in Biomedical Research. His research tries to understand the initial steps of sexual conversion and its epigenetic regulation.

Elisabet Tintó-Font is a PhD Student at ISGlobal. She holds a BSc in Biomedicine and a MSc in Microbiology. Her ongoing research is focused on the transcriptional response to heat shock in malaria parasites.

KEY POINTS

- Heritable transcriptional differences between malaria parasites at the same stage of life cycle development can have a genetic or epigenetic basis.
- Malaria parasites can also produce directed protective transcriptional responses in reaction to changes in their environment.
- Stochastic and spontaneous processes play an important role in generating transcriptional variation in malaria.
- Transcriptional plasticity plays a major role in the adaptation of malaria parasites to changes in their environment.

KEYWORDS

Malaria; Plasmodium falciparum; adaptation; transcriptional variation; bet-hedging; epigenetics.

ABSTRACT

Transcriptional differences enable the generation of alternative phenotypes from the same genome. In malaria parasites, transcriptional plasticity plays a major role in the process of adaptation to fluctuations in the environment. Multiple studies with culture-adapted parasites and field isolates are starting to unravel the different transcriptional alternatives available to *Plasmodium falciparum* and the underlying molecular mechanisms. Here we discuss how epigenetic variation, directed transcriptional responses, and also genetic changes that affect transcript levels can all contribute to transcriptional variation and ultimately parasite survival. Some transcriptional changes are driven by stochastic events. These changes can occur spontaneously, resulting in heterogeneity within parasite populations that provides the grounds for adaptation by dynamic natural selection. However, transcriptional changes can also occur in response to external cues. A better understanding of the mechanisms that the parasite has evolved to alter its transcriptome may ultimately contribute to the design of strategies to combat malaria to which the parasite cannot adapt.

INTRODUCTION

Differences among the individuals of a microbial population are essential for their survival in changing environments, as diversity provides the grounds for dynamic natural selection when the conditions of the environment fluctuate. Similar to bacteria or other parasitic protozoans, human malaria parasites are exposed to fluctuations in the conditions of the environment in which they can establish long-term infections (i.e. the human blood). This include changes in temperature associated with fever episodes, changes in nutrient availability associated with the metabolic or nutritional state of the host, changes in erythrocyte properties associated with host genetic traits, or changes in host immunity, among others.

Phenotypic diversity within a population implies that, under a given environmental condition, not all individual cells have optimal fitness and some cannot survive. Optimal fitness of the majority of individuals is favored in some multicellular higher eukaryotes such as humans, but it is of little importance in microbial communities with population sizes several orders of magnitude higher. Instead, if unpredictable changes in the environment occur, the population will prevail only if it contains some cells that can survive. As a matter of fact, in microbial communities natural selection largely operates at the level of the population, rather than at the individual cell level, and the level of within population diversity is a selectable trait [1]. In the case of malaria parasites, this implies that blood infections may not consist of populations in which all individual parasites have optimal fitness, but rather populations with an optimal level of diversity that enables survival under different conditions, even if it is at the cost of losing many individual cells at each generation.

Natural selection operates on phenotypes, rather than directly on genotypes. Selectable phenotypes can be determined by differences in the primary sequence of the genome, but also by other mechanisms. In this review we will focus on transcriptional variation in malaria parasites, which typically results in differences in protein expression and subsequently in phenotypes. As described in detail below,

different molecular mechanisms can lead to transcriptional differences in malaria parasites. Differences can arise spontaneously and stochastically in a population before any change in the environment occurs, but transcriptional alterations can also occur as a response that follows an external cue. Most of this review will focus on *Plasmodium falciparum*, which is responsible for the vast majority of severe human malaria cases, because transcriptional variation in other *Plasmodium* species has not been studied in detail.

An important consideration is that the main source of transcriptional differences in malaria parasites is by far life cycle progression [2-5]. Parasites at different stages of development have different needs and use their genes differently. Here we will only focus on transcriptional differences between parasites at the same stage of life cycle development.

TRANSCRIPTIONAL VARIATION AS A CONSEQUENCE OF CHANGES IN THE PRIMARY DNA SEQUENCE

Genetic variability is commonly observed in *P. falciparum*, including single nucleotide polymorphisms (SNPs), small indels and major genomic changes such as gene copy number polymorphisms (CNPs) or translocations [6-9]. Some mutations result only in changes in the primary sequence of the protein products encoded, but others, discussed below, affect transcript levels (Fig. 1A).

An extreme case of genetic changes resulting in transcriptional alterations is the deletion of a gene. If a gene is deleted, its transcripts are not produced. Deletion of large subtelomeric regions including several genes are commonly observed in culture-adapted parasite lines. The genes involved play important roles in processes such as cytoadherence or gametocyte production that are essential in natural infections but dispensable *in vitro* [10-12]. In addition to deletions, CNPs observed in *P. falciparum* also include amplifications [6, 7, 13-15]. Generally there is a clear correlation between gene copy number and transcript levels [12], as exemplified by a duplication affecting 17 genes in chromosome 10 that resulted in

approximately twofold higher transcript levels of the majority of the genes [5]. Increased gene copy number is commonly observed for genes involved in drug resistance such as *pfmdr1, pfgch1*, *dhodh* or plasmepsins [12, 15-19].

In addition to transcriptional alterations occurring by outright deletion or amplification of a gene, genetic changes occurring in regulatory regions such as promoters also have the potential to affect transcript levels. This has been observed in *P. falciparum*, as exemplified by a deletion in the regulatory region of the *pfmrp2* gene (encoding an ABC transporter) that results in expression of the gene at later stages of the asexual blood cycle than in normal parasites, and confers drug resistance [20]. The effect of CNPs on the expression of genes outside the region affected has also been reported, pointing to the presence of regulatory DNA elements or genes encoding regulatory factors within the amplified or deleted regions [21, 22]. The characterization of genetic changes (CNPs or SNPs) and transcript levels in the progeny of a genetic cross [22] and in field isolates [23] led to the identification of regulatory hotspots, but the putative regulatory elements involved have not yet been characterized in detail. Chromosomal translocation of subtelomeric genes as a result of ectopic recombination events also has transcriptional consequences, as the expression of the genes involved is altered [9, 24]. Lastly, SNPs in the coding sequence of transcriptional regulators can result in altered transcription of their target genes. This situation has been described in malaria during culture adaptation [25] and in stable culture-adapted lines [26, 27]. In both cases, premature stop codons occurred in ApiAP2 transcription factors that are not essential for asexual growth such as PfAP2-G, which is the master regulator of sexual conversion. In this transcription factor, non-sense mutations result in inability to activate the gametocyte-specific transcriptional programme [27].

TRANSCRIPTIONAL VARIATION REGULATED AT THE EPIGENETIC LEVEL

Epigenetics refers to heritable differences between cells that cannot be explained by differences in the primary DNA sequence. Some malaria genes, termed clonally

variant genes, can be found in either an active or a silenced state in genetically identical parasites at the same stage of life cycle progression. Once established, the transcriptional state is stably transmitted from one cellular generation to the next in the absence of external cues, providing one of the best examples of *bona fide* epigenetic inheritance in any organism [28] (Fig. 1B). *P. falciparum* clonally variant genes include genes involved in antigenic variation [29], which contribute to immune evasion, and genes that confer different phenotypes for processes such as developmental fate determination, erythrocyte invasion or solute transport. Clonally variant genes have been identified by comparative transcriptional analysis in gene-centered studies [27, 30-36] and at a genome-wide level [5, 31, 37, 38], and can also be predicted from epigenomic profiles [39-41] or from comparative epigenomics [42, 43]. The genes and gene families that show clonally variant expression and their putative functions have been extensively reviewed elsewhere [44-47] and will not be further discussed here. The model that has emerged postulates that a limited number of gene families and individual genes show clonally variant expression as an intrinsic property, such that the genes of these families are found in different transcriptional states among individual cells of an isogenic population. The intrinsic heterogeneity in the expression of these genes, which precedes unpredictable environment changes, results in phenotypic variation within parasite populations, providing a substrate for natural selection.

A relevant concept for malarial clonally variant gene expression is bet-hedging, which refers to an adaptive strategy that relies on pre-existing diversity within populations. While bet-hedging is formally defined by mathematic descriptors of population fitness, conceptually it refers to a risk-spreading survival strategy involving stochastic but stable epigenetic heterogeneity within populations to ensure survival in changing environments, thus increasing overall population fitness [48-52]. Clonally variant expression in malaria has been proposed to form the basis of a bet-hedging adaptive strategy [5, 53]. However, the adaptive potential of transcriptional variation has been formally demonstrated only for the extensively studied *var* genes, involved in cytoadherence and immune evasion

(reviewed in [29, 54]), and for *clag3* genes involved in the transport of solutes, including nutrients and toxic compounds [55-58]. It is predicted that different families of clonally variant genes enable adaptation to different fluctuations in the human blood environment, but the cognate changing condition is not known for most variant families.

Molecular basis of clonally variant gene expression

The basic principles of the mechanism that regulates clonally variant expression of different *P. falciparum* gene families have been described. The regulatory regions of these genes lie within bistable chromatin domains [59], in which both an euchromatic and an heterochromatic state can be adopted and stably transmitted (Fig. 1B). The silenced (heterochromatin) state is characterized by tri-methylation at histone H3 lysine 9 (H3K9me3), whereas the active (euchromatin) state is characterized by acetylation at this same position (H3K9ac) [60-64]. The epigenetic memory of the active or silenced state of a variant gene is carried in the chromatin of the locus itself: histone modifications that determine the active or silenced state are maintained throughout the full asexual cycle including stages at which the gene is not expressed [42, 62-64]. However, while both states can be stably inherited for several generations, low-frequency switches from one state to the other occur in some parasites [32, 45]. In addition to alternative chromatin states, variant gene expression in *P. falciparum* involves relocation between different sites within the nucleus, at least for some gene families [40, 65-68].

Proteins that contribute to the establishment and maintenance of H3K9me3, such as H3K9-specific histone methyltransferases and deacetylases, as well as heterochromatin protein 1 (HP1), are necessary for the silenced state of clonally variant genes, whereas H3K9 histone demethylases and acetyltransferases enable the active state [40, 44, 47, 69, 70]. On top of this general mechanism common to all clonally variant genes, different histone modifications and enzymes control the variant expression of specific variant gene families such as *var*, which shows mutually exclusive expression [29, 71-76].

While the general mechanism regulating clonally variant expression has been elucidated, many questions still remain. One unresolved issue is how the transcriptional state of each individual variant gene is regulated independently of its neighbor genes [5, 62, 77, 78]. It is well-established that heterochromatin has the capacity to spread, both in model organisms [79] and in *P. falciparum* [80, 81], implying that independent heterochromatin-based regulation of neighbor genes requires a system to limit spreading. In other organisms this is achieved by boundary elements or insulators [82, 83], but in malaria parasites such elements still remain to be identified. Another as yet unanswered fundamental question is what makes a malarial gene clonally variant. Clonal variation implies the capacity to nucleate heterochromatin and to switch from one epigenetic state to another, but the determinants for these capacities remain unknown. The genomic location may play a role, as many clonally variant genes are located in subtelomeric regions and therefore could be influenced by the heterochromatin environment of the telomeres [84]. However, subtelomeric location is not strictly necessary for variant expression because many clonally variant genes are located outside of these regions [5, 39, 40]. Therefore, it is likely that the capacity to reversibly form heterochromatin is an intrinsic property of the primary sequence of the regulatory regions of variant genes. Either a specific sequence motif (e.g. the cognate motif of a specific transcription factor) or a particular DNA conformation [85] could be recognized by factors able to recruit the complexes involved in nucleating heterochromatin assembly, as described in other organisms [86, 87], but none of these elements has been identified in *P. falciparum* so far. In other eukaryotes the main trigger of H3K9me3-based heterochromatin formation depends on siRNAs and the RNA-induced transcriptional silencing (RITS) complex [79, 88], but they are absent from the *P. falciparum* genome [89].

Mechanisms of switching

Yet another major gap in our molecular understanding of clonally variant gene expression is the mechanism mediating switches between the two possible

chromatin states. Transcriptional heterogeneity develops spontaneously in parasite populations growing in an apparently homogeneous environment (e.g. in the same petri dish). This suggests that epigenetic switches can occur spontaneously as an intrinsic property of some genes [5, 32], notwithstanding the possibility that in some cases external cues may induce epigenetic changes. The on and off switching rate also appears to be a stable intrinsic property of a clonally variant gene, although this has only been studied in detail for *var* genes [90-93] and indirectly for *pfap2-g* [27].

Stochastic processes, which are an intrinsic feature of bistable systems and bet-hedging in many unicellular organisms [51, 52, 94-97], likely play a key role in variant gene switching in malaria. Intuitively, it is clear that when two identical cells are under an identical environment but a given locus switches in only one of them, a stochastic or probabilistic event is involved. It is important to keep in mind that a stochastic event has unpredictable consequences for an individual parasite, but at the population level it can result in a well-defined outcome such as a stable frequency of switching (Fig. 2). A stochastic event is defined as a process that cannot be predicted from the variables that can be quantified. All biological processes are subject to stochasticity despite being controlled by physical laws [98]. In gene expression, a process often based on a small number of molecules [1], the stochastic variability of the system or noise can arise from variation in the location and concentration of regulatory proteins (extrinsic noise) and from variability within the biochemical process itself (intrinsic noise) [99]. For the switching of clonally variant genes, located in bistable chromatin domains [62], stochastic interactions with histone modifiers and positive feedback loops can result in switching events [59].

We propose two distinct molecular mechanisms involving stochastic events that may promote switches between the two possible chromatin states of clonally variant genes: i) changes in the levels of the epigenetic regulators controlling

clonally variant genes; ii) errors in the transmission of the epigenetic memory during mitosis.

The former mechanism involves variation in the expression levels of epigenetic regulators within parasite populations, such that in some individual cells expression of the regulators is altered and this affects the dynamic equilibrium at clonally variant loci, resulting in a switch (Fig. 3A). Given that switching events are infrequent, cells with altered levels of the epigenetic regulators are expected to be similarly rare; hence, detecting them experimentally poses a major challenge. However, some reports described variant levels of epigenetic regulators among parasite isolates. This suggests that rather than rare transient alterations in the expression of the regulators (only in parasites in which a switch occurs), parasites with stable alterations in regulator levels are selected under some conditions. In this scenario, regulator levels mediate not only the switch but also the maintenance of specific epigenetic states. One study observed increased transcript levels of *P. falciparum* sirtuins in patients with severe malaria, and this was associated with increased expression of specific *var* gene subgroups [100]. Sirtuins are NAD+ dependent histone deacetylases that can sense the NAD+/NADH levels and therefore link the metabolic state of a cell with chromatin states [101, 102]. In *P. falciparum*, sirtuins have been proposed to play roles in *var* regulation and in adjusting multiplication rates [66, 84, 103, 104]. Another study observed altered transcript levels of the histone deacetylases HDAC1 (downregulated) and HDA1 (upregulated) in parasites from areas of low transmission intensity, and this was associated with increased expression of *pfap2-g* [105]. The authors proposed that the expression patterns of these enzymes determine the level of parasite investment in transmission. Reduced expression of genes encoding histone methyltransferases in severe malaria patients has also been reported [106]. However, no clear evidence for clonally variant expression of epigenetic regulators in culture-adapted parasites has been observed. Further studies will be needed to confirm if differences in the expression of epigenetic regulators play a role in determining switching events or stable transcriptional differences between parasite

populations. Nevertheless, this mechanism is unlikely to be the main determinant of epigenetic switches: the relatively small number of histone modifying enzymes identified in the parasite genome [107, 108] indicates that the same enzymes likely operate on all clonally variant loci, rather than each variant gene family being regulated by specific epigenetic regulators. Thus, if changes in the expression of epigenetic regulators were the main determinant of switches, such changes would be expected to have general effects on the expression of all variant genes, but this situation has not been observed.

The second possible mechanism that we propose for the switches in variant gene expression involves “controlled errors” in the transmission of the epigenetic memory during mitosis (Fig. 3B) [94, 95]. During DNA synthesis parental histones are sequentially removed and later assembled on the two daughter strands. This implies that cells have a mechanism to restore the epigenetic status in the two new DNA copies [109-111]. In *P. falciparum*, which divides through mitosis during growth in the human blood [112], errors in the transmission of histone modification patterns in bistable chromatin domains may underlie variant gene switches. Of note, errors are both common and necessary in biological processes as they enable diversity and the evolution of organisms. While epigenetic information can be transmitted from one generation to the next, its transmission through mitosis is far less faithful than the transmission of the primary DNA sequence by DNA polymerase I. As a consequence, epigenetic processes are more dynamic and better suited to generate plasticity and mediate adaptation to fluctuating conditions than genetic changes. The role of epigenetic memory transmission errors in *P. falciparum* clonally variant gene expression switches remains to be experimentally demonstrated.

SENSING PLUS DIRECTED TRANSCRIPTIONAL RESPONSES

The fundamental difference between the mechanisms that result in transcriptional variation described above and directed transcriptional responses lays in the dependence on external cues. Both mutations and epigenetic switches occur

spontaneously; they can confer a fitness advantage under unpredictable host conditions and mediate adaptation by natural selection, but they occur before the change in the environment. In contrast, a directed transcriptional response follows an external cue, involving sensing the external condition, signal transduction and changes in the expression of genes that mediate protection against the new condition and ultimately parasite survival (Fig. 1C). The response is in principle transient and non-heritable, but it remains possible that in some cases the directed response may involve alterations in the epigenetic state of a gene that are maintained and inherited after the external cue disappears. Such situation would include features from both spontaneous epigenetic variation and transient protective responses (Fig. 1B-C, bottom). However, no clear example of this situation has been described so far, so it will not be further discussed.

The ability to sense the environment and respond at the transcriptional level is widespread in prokaryotic and eukaryotic cells. However, theoretical models predict that for organisms that live in relatively stable environments bet-hedging-like adaptive strategies may be more cost-effective than sensing plus directed response systems [113]. Malaria parasites can sense changes in the environment and respond to them during stage transitions, as in the case of gametocyte activation when parasites are taken from the human circulation during a mosquito bloodmeal [114, 115]. However, this response does not appear to primarily occur at the transcriptional level. For many years, whether or not malaria parasites are able to produce directed protective transcriptional responses in reaction to fluctuating conditions within the same host has remained controversial, and some authors proposed that *P. falciparum* blood stages have a hard-wired transcriptome unable to respond to changes in their environment [116-118]. Other authors described transcriptional responses following some challenges [116, 119-128], although in most cases they were of low magnitude compared to the responses commonly observed in other microorganisms. Additionally, the link between the transcriptional alteration and protection often remained unclear, and in some cases

the transcriptional changes observed may reflect parasite death or delayed life cycle progression rather than a protective response.

Two recent reports have unambiguously demonstrated that malaria parasites have the capacity to produce protective transcriptional responses to changes in their environment. In one case it was shown that *P. falciparum* can sense the depletion of a specific serum component, lysophosphatidylcholine, and respond by activating the expression of *pfap2-g* (possibly via GDV1 [129]), which results in sexual conversion [130]. This study reveals intricate links between epigenetic regulation and directed transcriptional responses, as the *pfap2-g* locus is silenced by an epigenetic heterochromatin-based mechanism in asexually-growing parasites [27]. However, the new epigenetic state is not inherited because it triggers a stage transition. In addition, lysophosphatidylcholine depletion resulted in changes in the expression of many metabolism-related genes, demonstrating the capacity of the parasite to mount a protective transcriptional response to adapt its metabolism to the host conditions, besides the developmental transition. The other report shows that the murine malaria parasite *P. berghei* (and possibly also *P. falciparum*) can sense nutrient restriction and, after signaling by the KIN protein kinase, activate a transcriptional program that results in reduced multiplication rates [131]. These two studies clearly demonstrate that malaria parasites are able to sense fluctuations in their environment and respond at the transcriptional level, beyond developmental transitions. However, the master transcriptional regulators driving the metabolic readjustment upon lysophosphatidylcholine depletion or calorie restriction remain to be identified. Future research should also determine to which other fluctuating conditions parasites can produce directed transcriptional responses.

TRANSCRIPTIONAL VARIATION IN PARASITES PRODUCING HUMAN MALARIA INFECTIONS

Transcriptional variation has also been characterized in natural malaria infections, but studying the transcriptome of field isolates poses several important challenges. First, parasite densities in naturally infected individuals are usually very low,

limiting the amount of parasite RNA that can be obtained from a blood sample. Moreover, only ring-stages and mature gametocytes are present in the circulation, which makes culturing indispensable to study the transcriptome of other blood stages. Thus, maturing parasites ex vivo to obtain parasites at late asexual stages or culturing for a small number of cycles to amplify the amount of parasite material are common practice. This requires the availability of a parasite culture facility near the collection site. Additionally, culturing for a few cycles complicates the interpretation of the results because it can have an impact on several parasite traits [132] after only a few days, as shown for *var* and *clag3* transcriptional patterns [56, 133, 134]. Indeed, field isolates show important transcriptional differences with stable culture-adapted lines, mainly in genes involved in immune evasion, cytoadhesion, erythrocyte invasion and transmission [21, 38, 135-139]. However, direct transcriptional analysis without culturing restricts the study to genes expressed in ring and mature gametocyte stages, and there are differences between isolates in the precise age of the parasites or in the abundance of gametocytes. Stage differences have been proposed to be a major confounder in studies on transcriptional variability between field isolates [23, 135, 136, 140]. Lastly, the extensive genome polymorphism observed in subtelomeric regions among field isolates is also a major hassle for their transcriptional analysis [9]. In spite of these limitations, several studies have attempted to characterize field isolates at the transcriptional level, providing important initial insight both for specific gene families and at the full genome level.

Many studies have attempted to identify associations between specific parasite transcriptional profiles and malaria clinical presentation. Genome-wide studies of field isolates revealed altered expression of virulence genes in patients with cerebral malaria compared to asymptomatic or uncomplicated malaria patients, or between patients with different levels of parasitemia [106, 136, 137, 141, 142]. These included genes that mediate host cell remodeling, immune evasion or cytoadherence, among others. Genome-wide studies also identified different transcriptional states in *P. falciparum* ring stages that appear to reflect different

parasite metabolic states [143]. Some of the genes involved, which are not clonally variant, are related to central carbon metabolism. Alternative transcriptional states were proposed to reflect different nutrient availability and a stress response associated with host clinical manifestations such as fever or inflammation [143], and later found to be associated with parasite load [142] or with malaria severity [106]. Specific transcriptomic profiles associated with infection in children [144] or with placental malaria [145] have also been reported.

Studies of transcriptional variation among field isolates centered on specific variant gene families have focused mainly on genes related to erythrocyte invasion or immune evasion and cytoadherence. *P. falciparum* can use alternative invasion pathways determined by the expression of clonally variant genes of the families erythrocyte binding-like (*eba175*, *eba140*, *eba181* and *ebl1*) and reticulocyte binding-like homolog (*rh1*, *rh2a*, *rh2b*, *rh4* and *rh5*) [146, 147]. Field isolates exhibit different invasion phenotypes and vary in their expression of these invasion genes, but no clear association with clinical manifestation has been identified [148-155]. Whether the driving force for the variant expression of *eba* and *rh* genes in the field is functional diversification, immune evasion, or both still remains to be determined [147, 156]. The expression of variant genes involved in immune evasion and cytoadherence has also been extensively studied in field isolates. Strong host antibody responses are associated with lower total expression of *var* genes [157], and expression of specific *var* genes or subsets of genes are associated with clinical presentation. The best know example of such associations is expression of *var2csa*, which is linked to CSA binding and placental malaria [158, 159]. Other types of severe malaria are generally associated with expression of *var* genes of group A and/or containing the domain cassettes DC8, DC13 or DC6 [54, 160-164]. These associations were also observed in a recent study using RNA-seq analysis of field isolates and de novo *var* gene assembly, which also identified additional *var* genes linked to severe disease [106]. In the case of *var* genes with DC8 or DC13, the link with severity involves binding to the endothelial protein C receptor (EPCR) [165, 166].

An important remaining question for all studies attempting to correlate parasite transcriptional patterns with malaria clinical presentation is about the direction of causality: it is currently unclear which transcriptional patterns cause severe disease, and which are the consequence of parasite adaptation to host responses associated with disease severity such as fever or inflammation. Future research will need to address this question, confirm the associations observed and ideally provide mechanistic insight.

Transcriptional profiles have also been studied to compare parasites from regions with different malaria epidemiology or drug resistance status. A recent study suggested that transmission intensity influences parasite transcriptomes. Parasites from high-transmission areas generally presented transcriptional patterns consistent with higher investment in asexual replication and less in transmission compared to parasites from low-transmission areas [105]. Drug usage may also shape parasite transcriptomes. While mutations are the main determinant for antimalarial drug resistance [167], a recent study identified transcriptional alterations associated with artemisinin resistance. The transcriptional signature of resistant parasites involved delayed progression through the ring stage and upregulation of the unfolded protein response, which contributes to overcome the damaging effect of the drug [168].

Altogether, it is now clear that transcriptional patterns *in vivo* are affected by many factors, including the host environment and the epidemiological context. However, the relative contribution of genetic and epigenetic changes to transcriptomic heterogeneity in field isolates still remains to be determined for many of the examples discussed. While invasion ligand or *var* gene switches appear to have a clear epigenetic basis, it is likely that stable transcriptional adaptations such as those observed in artemisinin-resistant parasites [168], mostly affecting genes that are not clonally variant, have a genetic basis. Studies coupling transcriptomic analysis with full genome sequencing of field isolates are starting to define the

mechanistic basis of transcriptional variability in the field [23]. As a complement to studies with field isolates, parasite transcriptional patterns can also be studied *in vivo* in controlled human malaria infection (CHMI) studies. This approach has the advantage of a homogeneous parasite genetic background. Studies focused on *var* and *clag3* genes already proved the usefulness of this approach, demonstrating a reset of the epigenetic memory during transmission stages [56, 169-172].

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Multiple studies have unambiguously established that two malaria parasites at the same stage of development can have substantially different transcriptomes, which plays an important adaptive role. However, a deeper characterization of transcriptional variation in malaria is complicated by the limitations of methods that use bulk population analysis to study properties that vary from cell to cell. To overcome this limitation, several studies have analyzed subclones recently obtained from already clonal parasite lines, because transcriptional patterns are maintained for the few cycles between subcloning and analysis [5, 31, 37]. This approach provides two major advantages over regular bulk population analysis: first, transcriptional patterns can be compared between parasites of the same genetic background. Second, it captures transcriptional heterogeneity within a parasite population (Fig. 4A). However, this is a time-consuming approach not suitable for the identification of rare transcriptional states that would require the analysis of large numbers of subclones, or for the characterization of field isolates.

The recent development of single-cell transcriptomics for malaria parasites is a major breakthrough in the field that holds the promise of overcoming these difficulties and boosting our understanding of transcriptional variation in malaria [173-177]. Single-cell RNA-seq enables the simultaneous characterization of the transcriptome of thousands of individual parasites (Fig. 4B). Importantly, it is suitable for the analysis of field isolates and in principle it can be applied to study transcriptional variation in any *Plasmodium* species (even those that cannot be cultured *in vitro*). Currently, the number of transcripts detected per cell is relatively

low, implying that transcripts from genes expressed at low level are either undetected or detected with a number of reads that precludes quantitative analysis. This restricts the analysis of cell to cell transcriptional variation to abundantly expressed genes, but it is expected that technological improvements will soon enable the meaningful analysis of more genes. Even with the current limitations, single-cell RNA-seq and single-cell qPCR have already provided important new insight on sexual commitment [173, 176-178], identified new male and female gametocyte markers [175], and challenged the previous dogma in the field of a continuous cascade of gene expression [174]. Of note, the latter study also included analysis of *P. berghei*, demonstrating the applicability of the method to different malaria species.

Important insight has been gained on the molecular mechanisms that generate transcriptional variation in malaria parasites, including the molecular basis for stochastic epigenetic variation. Protective directed transcriptional responses have also been unambiguously identified. However, as noted through the text, many questions still remain. For instance, some transcriptional changes observed in the field, such as those associated with alternative metabolic states or with artemisinin-resistance [143, 168], cannot be unambiguously ascribed to any of the three types of transcriptional variation that we describe. For convenience, we classify transcriptional variation in these three types, but they are likely interrelated. Also important to note, for much of this review for simplicity we have referred to the active or silenced state of clonally variant genes, but it is likely that intermediate states exist. We have neither discussed transient non-heritable fluctuations in gene expression levels, because these scenarios have not been explored yet in malaria. Future studies combining accurate transcriptomic, epigenomic and genome sequence analysis with single-cell technologies should move the field forward and identify all the strategies available to the parasite to change its transcriptome to adapt to changes in its environment, including human interventions. Ultimately, we should be able to unravel the full adaptive potential of transcriptional variation in

malaria parasites to guide the design of new interventions that are not susceptible to failure by parasite adaptation.

ACKNOWLEDGMENTS

We apologize to colleagues whose work we could not discuss or cite because of space constraints. The icons used in some figures were obtained from https://smart.servier.com (Cellular Biology, Intracellular Components Section) under Creative Commons Attribution 3.0 Unported License. This work was supported by grant SAF2016-76190-R to A.C. from the Spanish Ministry of Economy and Competitiveness (MINECO)/ Agencia Estatal de Investigación (AEI), co-funded by the European Regional Development Fund (ERDF, European Union); O.L.-B. is supported by a FPU fellowship from the Spanish Ministry of Education, Culture and Sports (FPU014/02456). E.T.-F. is supported by a fellowship from MINECO (BES-2014-067901), co-funded by the European Social Fund (ESF), European Commission. ISGlobal is a member of the CERCA Programme, Generalitat de Catalunya.

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FIGURE LEGENDS

Figure 1. Sources of transcriptional variation. Transcriptional differences between malaria parasites can arise from spontaneous changes both at a genetic (**A**) or epigenetic (**B**) level, or can occur as a response to an external cue (**C**). In some cases, responses to external cues may result in an epigenetic alteration, a mechanism that would share features of both B and C (box at the bottom). SNP: single nucleotide polymorphism; CNP: copy number polymorphism.

Figure 2. Predictability of stochastic processes. Although stochastic processes are random, the outcome is predictable when the constraints of the system are known and the number of events is large. As an example, the result of rolling a dice a single time is unpredictable, whereas the result of rolling a dice 10^9 (a billion) times can be accurately predicted. A typical human malaria blood infection can contain 10^9 parasites and even many more. Stochastic switching between active and silenced chromatin states at malaria variant genes results in a controlled

outcome, such that heterogeneity in the population is constantly provided at the same rate.

Figure 3. Possible mechanisms of switching. A, The epigenetic switch of clonally variant genes can be driven by variation in the levels of histone-modifying enzymes due to heterogeneity within the population or induced by external cues. Red flags are H3K9me3 silencing marks. Green flags are H3K9ac active marks. HDMs and HMTs are histone demethylases and methyltransferases, respectively, that operate on H3K9; HATs and HDACs are histone acetyltransferases and deacetylases, respectively. **B**, Switching between euchromatin and heterochromatin can occur by controlled errors in the transmission of the epigenetic memory. During DNA replication, histone chaperones interact with the DNA helicase minichromosome maintenance protein complex (MCM) to disassemble the nucleosomes of the parental DNA molecule. Parental histones, depicted in pale brown, are randomly distributed between the two daughter strands through the interaction of histone chaperones with the DNA clamp proliferative cell nuclear antigen (PCNA), which is responsible for maintaining the processivity of the DNA polymerases in both the leading and the lagging strand [179, 180]. Newly synthesized histones, depicted in green, are also assembled into the daughter strands. In order to maintain the epigenetic memory, the newly synthesized histones need to be modified to restore the original epigenetic state of the locus. This is driven by the presence of 'bookmarking' factors that recruit the histone modifying enzymes [109, 110]. In this example a histone methyltransferase (HMT) is recruited to maintain the repressed state of the locus. Errors in the maintenance of the epigenetic memory during mitosis can result in switches between clonally variant gene states.

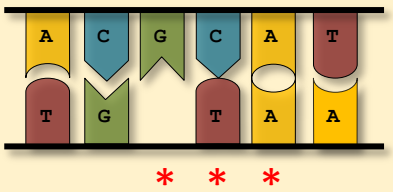
Figure 4. Methods to study cell to cell transcriptional variation. To overcome the limitations of studying bulk populations, a transcriptionally heterogeneous population can be either subcloned and analyzed soon thereafter, when the population arising from a single parasite is still relatively homogeneous (**A**), or

analyzed directly by single-cell technology (in this example, single-cell RNA-seq) to obtain information for each parasite individually (**B**). Colors represent different transcriptional patterns.

Fig. 1.

A

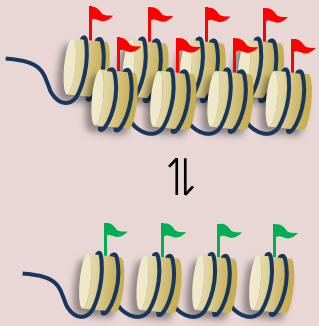
GENETIC



- SNPs, indels or major rearrangements (e.g. CNP)
- Variation precedes changes in the environment
- Slow, long-term adaptation to new conditions
- ‘Irreversible’
- Heritable
- Stable traits
- Adaptation by natural selection

B

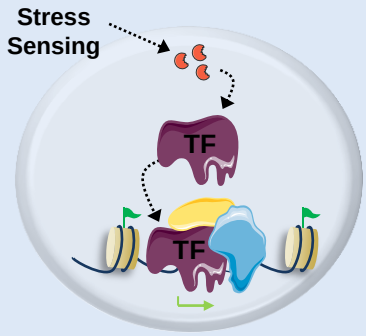
EPIGENETIC



- Switch between active and repressed chromatin state
- Variation precedes changes in the environment
- Fast adaptation to fluctuating conditions
- Reversible
- Heritable
- High plasticity
- Adaptation by natural selection

C

DIRECTED



- Directed transcriptional response to a stress
- Variation follows a change in the environment
- Immediate sensing-response mechanism
- Transient
- Non-heritable

DIRECTED + EPIGENETIC

- Directed transcriptional response to a stress, but resulting in an epigenetic, heritable alteration

Fig. 2.

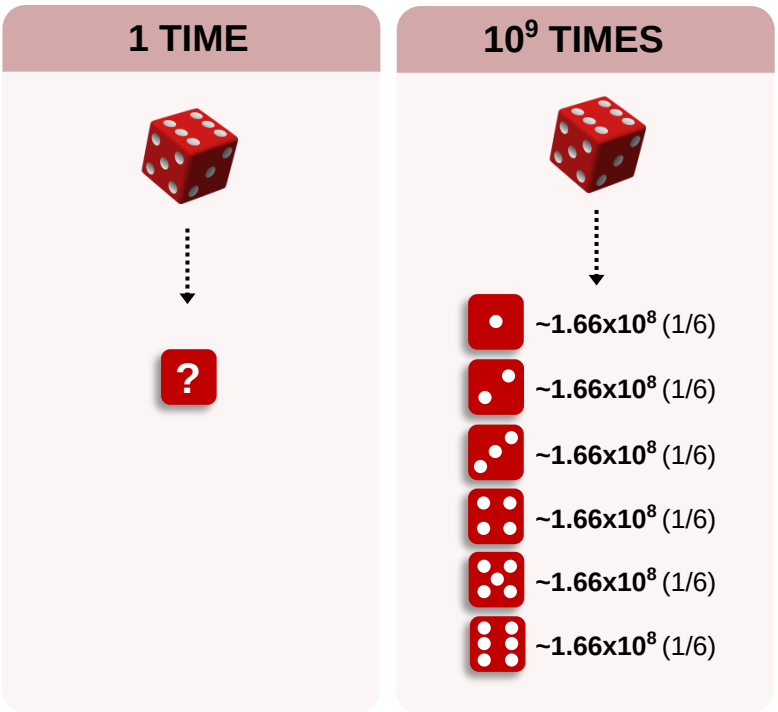
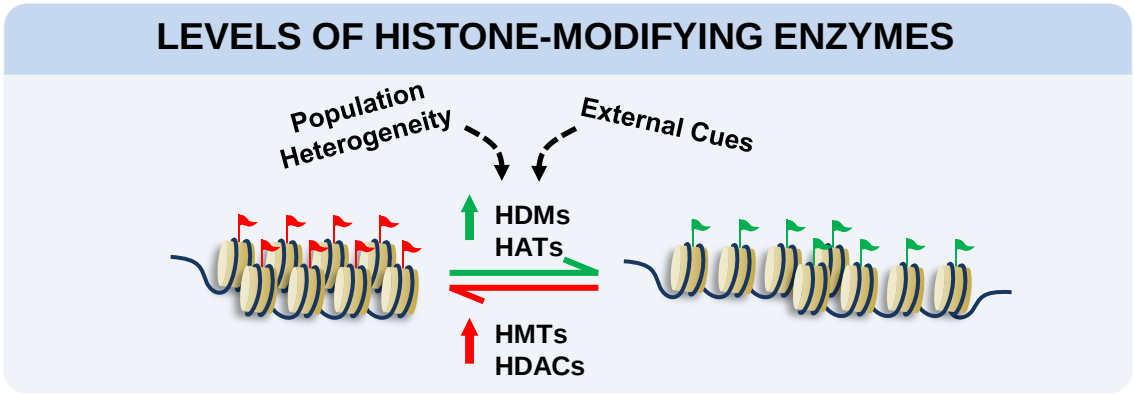

1 TIME
?
10^9 TIMES
~1.66x10^8 (1/6)
~1.66x10^8 (1/6)
~1.66x10^8 (1/6)
~1.66x10^8 (1/6)
~1.66x10^8 (1/6)
~1.66x10^8 (1/6)

Fig. 3.

A


LEVELS OF HISTONE-MODIFYING ENZYMES
Population Heterogeneity
External Cues
HDMs
HATs
HMTs
HDACs

B

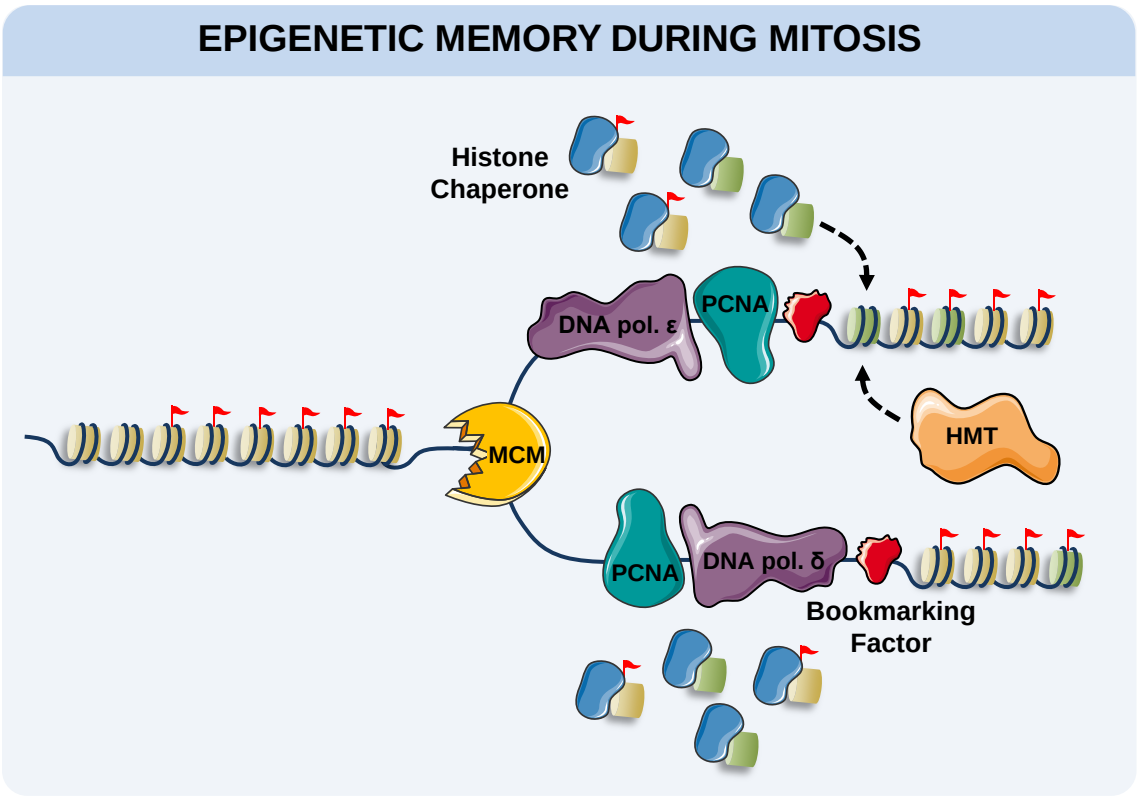
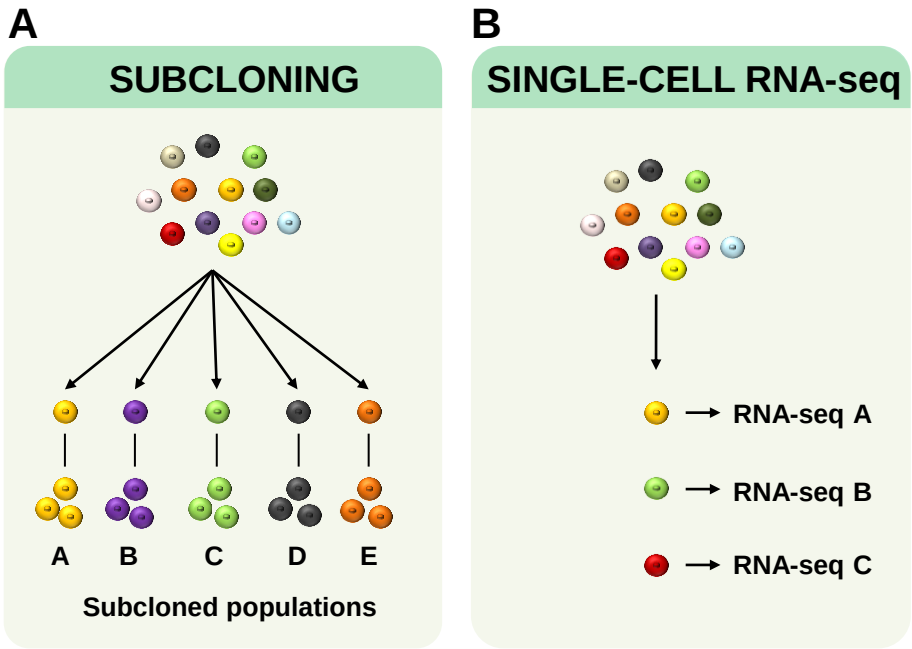

EPIGENETIC MEMORY DURING MITOSIS
Histone Chaperone
DNA pol. ε
PCNA
HMT
MCM
PCNA
DNA pol. δ
Bookmarking Factor

Fig. 4.


A
SUBCLONING
A
B
C
D
E
Subcloned populations
B
SINGLE-CELL RNA-seq
RNA-seq A
RNA-seq B
RNA-seq C