Malaria parasites do respond to heat

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ABSTRACT

The capacity of malaria parasites to respond to changes in their environment at the transcriptional level has been the subject of debate, but recent evidence has unambiguously demonstrated that *Plasmodium* spp. can produce adaptive transcriptional responses when exposed to some specific types of stress. These include metabolic conditions and febrile temperature. The *P. falciparum* protective response to thermal stress is similar to the response in other organisms, but it is regulated by a transcription factor evolutionarily unrelated with the conserved transcription factor that drives the heat shock (HS) response in most eukaryotes. Of the many genes that change expression during HS, only a subset constitutes an authentic response that contributes to parasite survival.

KEY WORDS

Plasmodium falciparum; adaptation; directed transcriptional response; heat shock response; fever; PfAP2-HS.

What is and what is not a response?

A response is something said or done directly as a reaction to another event. In biology, a response is a reaction of an organism to a change in its environment. Organisms have evolved multiple mechanisms to survive under the different environmental conditions that they encounter, but not all of these mechanisms are responses. When a frilled lizard feels threatened and spreads out the frill around its neck, it is a response. However, the sturdy skin of a rhino provides a survival advantage in its harsh savannah environment, but the animal always carries it, so it is not a response. A cow becomes malnourished and skinny, or eventually dies, when there is a severe drought that reduces food and water availability, but this is not a response: it is just a consequence of a condition of the environment, not an active reaction by the cow to counteract it. Although it could be argued that all the genetic adaptations occurring during the evolution of a species can be considered a slow response of the species to its environment, in this review we will not enter this philosophical debate and we will consider as responses only the direct reactions that occur in an individual organism immediately after a change in the environment.

At the molecular level, most organisms are able to produce directed, rapid responses when the conditions of their environment change and threaten their survival. Typically, the environmental condition itself or a cue are sensed, and after signal transduction a molecular pathway is activated. The response itself can consist in a large variety of molecular events, including transcriptional and other types of changes [1-3]. In all cases, for a molecular pathway to be considered a response, it needs to be activated or enhanced in reaction to a

change in the environment. Permanent molecular traits with which an organism is equipped are not a response, even if they are important for survival. A classic example of a molecular response is the activation of the *Escherichia coli* lactose operon when the medium contains low glucose and high lactose concentrations [4]. In the absence of lactose, the *lac* repressor prevents the transcription of the *lac* operon, which contains genes needed for lactose use. However, in the presence of lactose, a lactose-derived product inactivates the repressor, enabling transcription of the *lac* operon. Together with additional regulatory mechanisms, this transcriptional response enables using lactose when it is available, and to save resources when it is not.

Similar to other organisms, malaria parasites can respond to changes in their environment at the molecular level. A clear example is the activation of parasites at the gametocyte stage when they are ingested as part of a mosquito bloodmeal. The conditions in the mosquito vector, which entail a drop of temperature and pH and exposure to xanthurenic acid, among other changes, trigger a molecular cascade that leads to conversion into gametes [5]. However, this response is not regulated at the transcriptional level, and whether or not malaria parasites are able to produce **directed transcriptional responses** (see **Glossary**) was for some time controversial [6]. In this review, we will discuss recent evidence demonstrating that *Plasmodium* spp. can produce protective transcriptional responses to specific changes in their environment. We will mainly focus on the parasite response to febrile temperatures.

The mode of life of malaria parasites: coping with changes

Plasmodium spp. have a complex life cycle that involves development in different hosts and tissues, including the mosquito midgut, hemolymph and salivary glands, and the vertebrate liver and blood. The **intraerythrocytic development cycle (IDC)** involves asexual multiplication within erythrocytes in the vertebrate blood. Repeated rounds of the IDC result in exponential growth and long-term infection. The IDC is responsible for all clinical symptoms of malaria, which in humans commonly include cyclical fever and headache, and in some cases more severe complications [7].

Malaria parasites have evolved mechanisms to bear with the different conditions that their complex life cycle entails. This is achieved mainly at the transcriptional level, such that the same genome translates into a different transcriptome at each stage of development [8-10], although post-transcriptional mechanisms also play a key role at some stages [11]. In addition to environmental changes associated with normal life cycle progression, parasites also face fluctuations in the conditions of the environment within each niche. For instance, blood stages are exposed to changes in nutrient availability, host immunity, drug exposure or temperature (during fever episodes), among others [12,13]. These changes do not occur in all hosts or at every round of the IDC, which makes them largely unpredictable for the parasite. Adapting to unpredictable fluctuations in the environment requires fundamentally different strategies from adapting to the predictable and sequential changes associated with life cycle progression. While a repertoire of transcriptomes specifically customized for each particular developmental stage is ideally suited to support life cycle progression, surviving unpredictable changes within each niche

requires alternative strategies, such as having plasticity within each stage-specific transcriptome.

To cope with changes or fluctuations in the conditions of the human circulation niche, malaria parasites use three main adaptive strategies: genetic changes, **bet-hedging** and directed transcriptional responses [14]. In both genetic changes and bet-hedging, diversity is generated spontaneously within the parasite population before a change in the environment occurs, enabling natural selection of parasites with high fitness when the environment changes. In contrast, direct transcriptional responses involve the activation of a new transcriptional program following a change in the environment. The main difference between genetic changes and bet-hedging is that the former relies on mutations in the primary sequence of the parasite DNA, which are largely irreversible, whereas the latter typically involves transcriptional changes regulated at the epigenetic level, which are heritable but reversible and highly dynamic. Low-frequency spontaneous transitions between euchromatic and heterochromatic states at clonally variant genes underly bet-hedging in *Plasmodium* spp. [15-18]. In ***Plasmodium falciparum***, the most lethal human malaria parasite species, antimalarial drug resistance [19] and antigenic polymorphism [20] are typically associated with genetic changes, whereas antigenic variation [21] and permeability changes to prevent the uptake of toxic compounds [22,23] are mediated by bet-hedging strategies [14]. However, the large number of genes that show clonally variant expression [18] or the associated heterochromatin marks [24,25], together with the large transcriptional heterogeneity observed at the onset of a human blood infection

[26], suggest that bet-hedging strategies likely play a major role in the adaptation of malaria parasites to many additional fluctuating conditions.

A directed transcriptional response typically involves sensing a new condition of the environment, followed by a signal transduction cascade that leads to activation of specific transcription factor(s), which drive transient and non-heritable gene expression changes. The response enables survival under the new environmental conditions [1-3] (**Figure 1**). For many years, it was unclear whether or not malaria parasites are able to produce directed transcriptional responses. While other organisms rapidly and massively activate or silence the expression of relevant genes as the conditions of the environment change, including metabolic, osmotic or thermal conditions, no well-defined responses were observed under similar conditions in *P. falciparum*. This led some authors to propose that *P. falciparum* may have a hard-wired transcriptome unable to respond to the conditions of its environment [27-29]. This opened a debate [6] that was settled by recent studies that unambiguously showed that malaria parasites can produce directed transcriptional responses when exposed to some specific external conditions. Here we will describe only three well-defined examples of directed transcriptional responses (**Figure 1**), but many other studies have reported transcriptional changes after exposing parasites to different stress conditions such as restriction of specific nutrients or exposure to drugs [30-35]. However, in many cases, the transcriptional changes occurred in genes involved in processes unrelated with protection from the stress conditions, and it was impossible to distinguish between authentic

transcriptional responses and changes in transcript levels attributable to parasite death or growth arrest.

A first example of a protective transcriptional response involves calorie restriction in the murine malaria parasite *P. berghei*. A serine/threonine kinase termed KIN was identified as a nutrient sensor that under nutrient-poor conditions is activated, which leads to reduced expression of genes related with parasite replication, resulting in reduced proliferation and virulence. While the nutrient sensor of this pathway was identified, the transcription factor(s) driving the gene expression changes were not [36]. The second example is the transcriptional response of *P. falciparum* to depletion of the serum lipid lysophosphatidylcholine (LysoPC), needed for phosphatidylcholine (PC) synthesis [37-39]. Depletion of LysoPC and downstream metabolites (e.g., choline) results in increased expression of genes encoding metabolic enzymes such as ethanolamine kinase (*ek*) and phosphoethanolamine N-methyltransferase (*pmt*) that enable PC synthesis by an alternative pathway. Additionally, LysoPC depletion results in increased sexual conversion [38], which is mediated by activation of the expression of the transcription factor *pfap2-g* [40] and its upstream regulator *gdv1* [41] in only a subset of cells [37,38]. The sensor of LysoPC levels and the transcription factor(s) driving the compensatory metabolic response have not been identified. The third example of a malarial directed transcriptional response is the *P. falciparum* response to febrile temperatures [42-47]. A key transcription factor driving this response was recently identified [43,48]. In the following sections, the response to heat stress will be discussed in more detail. The parasite responses to changes in

metabolic conditions, including calorie restriction and LysoPC depletion, have been recently reviewed elsewhere [13,49,50].

The examples described above clearly establish that *Plasmodium* spp. can actually produce protective transcriptional responses to cope with fluctuations in their environment, but only for a limited number of conditions. This fits well with the paucity of genes encoding specific transcription factors in malarial genomes, which in *P. falciparum* include 27 genes of the ApiAP2 family **(Box 1)** and very few additional genes [51-53]. This number of specific transcription factors may be just sufficient to orchestrate progression through the complex parasite life cycle and to respond to a few frequently encountered environmental fluctuations. It has been proposed that adaptation to diverse and unpredictable environmental changes that occur infrequently is more efficiently achieved by bet-hedging strategies, which do not require investing in costly sensing systems for each condition, whereas adaptation to frequently encountered conditions is achieved more efficiently using directed responses [54,55]. The observation that *P. falciparum* uses directed transcriptional responses when exposed to febrile temperatures, calorie restriction or low LysoPC levels fits well with this model: these are relatively common conditions associated with clinical malaria (febrile temperature), host malnutrition and high parasite density (calorie restriction and low LysoPC) [13,38,47,50].

Impact of febrile temperatures on *P. falciparum*: individuals die, the population survives

Periodic fever is the most characteristic clinical symptom of malaria disease in humans. It is triggered by schizont rupture, which releases into the bloodstream invasive merozoites together with other parasite-derived molecules often classified as “toxins” (**Box 2**). Schizont rupture is repeated at the end of each round of the IDC, giving rise to periodic fever episodes during which body temperature can reach >40ºC (**Box 3**). Fever is only triggered above a certain parasitemia, typically referred to as the pyrogenic threshold, which depends on multiple factors [47,56].

Fever episodes can be mimicked *in vitro* using a defined **heat shock (HS)**, consisting of incubation of the cultures at a temperature above 37ºC for several hours. Different research teams have used different temperature and duration for HS, because the large variability of natural malarial fever episodes makes it impossible to define a consensus set of HS conditions to mimic a malaria fever episode (**Box 2**). In spite of the diversity of HS conditions used, most studies reached similar conclusions about the effect of HS on parasite viability: thermal stress can kill *P. falciparum* blood stages, affecting late-stages (trophozoites and schizonts) more severely than ring stages [43,57-60]. Because of the differential sensitivity between developmental stages, fever may contribute to parasite synchronization [59,61,62]. In addition to producing cell death, which likely involves programmed cell death pathways [42,58,60], febrile temperatures can cause developmental arrest and delayed cell cycle progression [43]. One study reported that a HS during the ring-stage protects against subsequent HS later during the same round of the IDC [63]. Besides, HS results in increased

sexual conversion rates, possibly reflecting that the parasite invests more in transmission to mosquitoes to escape the thermal stress situation [46,64].

Of note, the impact of host fever on parasite viability in human malaria infections is not well understood [47]. Assuming that it is similar to the effect *in vitro*, it has been proposed that malaria fever may be advantageous not only for the host, but also for the parasite: reduction of parasitemia when it reaches the pyrogenic threshold may keep parasite density oscillating around an equilibrium value, facilitating host survival. This in turn would provide to the parasite population more opportunities for transmission and long-term survival [47,62,65].

In all organisms, a small temperature increase above their optimal growth temperature can lead to accumulation of misfolded and aggregated proteins [66]. Therefore, febrile temperatures are expected to represent an important challenge for the parasite proteome, even more for *P. falciparum* than for other organisms because many *P. falciparum* proteins contain aggregation-prone poly-asparagine repeats [67]. To protect cells from heat-induced damage, organisms in all kingdoms of life have an evolutionarily conserved **HS response** that involves the rapid activation of genes encoding **chaperones** and several other transcriptional changes. This results in an increase in the protein folding capacity of the cell, which helps to maintain **protein homeostasis (proteostasis)**, together with changes in processes such as metabolism, DNA repair or protein degradation [66,68]. The cytosol-based HS response is one of the major proteotoxic stress response pathways in eukaryotic cells, together

with the endoplasmic reticulum-based unfolded protein response (UPR) and the mitochondrial UPR (UPRmt) [3]. In *P. falciparum*, several genome-wide studies have reported major transcriptional alterations involving hundreds of genes in parasites exposed to HS, suggesting that malaria parasites can produce a HS response. Most of these studies identified upregulation of the expression of genes involved in protein folding, and specific studies identified changes in other processes such as host cell remodeling, metabolic pathways, signal transduction and mitochondrial processes [42-46]. However, because different studies used different conditions for the HS and different methodologies, it is impossible to define a consensus set of genes that change expression during HS (**Table S1**). It is likely that HS of different severity or at different stages of the IDC induces different transcriptional alterations. Furthermore, as often occurs when studying the response to stress conditions, a major challenge for these studies was to distinguish between transcript level changes that constitute the actual protective response and changes that reflect parasite damage or death produced by the HS.

PfAP2-HS and the *P. falciparum* protective HS response

The characterization of a parasite line that shows HS resistance heterogeneity among individual parasites [18] recently led to the identification of a transcription factor of the ApiAP2 family, termed PfAP2-HS, as the master regulator of the protective HS response in *P. falciparum* [43]. PfAP2-HS is a large protein (3,858 amino acids) characterized by the presence of three Apetala2 (AP2) domains near the C-terminus (D1-3) (**Figure 2**). Recombinant domain D1 was previously shown to recognize a DNA motif termed G-box

((A/G)NGGGG(C/A)) *in vitro*, with increased affinity when the motif occurs in tandem [69,70]. No *in vitro* DNA binding was observed for the other two AP2 domains (D2 and D3).

The function of PfAP2-HS was characterized using a suite of transgenic parasite lines in which PfAP2-HS was tagged or knocked out, and parasite lines with spontaneous truncations of the protein [43]. Transcriptomic analysis revealed that PfAP2-HS rapidly activates an extremely compact set of genes upon HS, which comprise the canonical *hsp70-1* and *hsp90* chaperone-encoding genes **(Box 4)**, and a third gene of unknown function (PF3D7_1421800). PfAP2-HS knockout parasite lines and parasite lines expressing truncated PfAP2-HS lacking D3 are unable to rapidly activate these genes upon HS and are much more sensitive to HS than wild type parasites, indicating that PfAP2-HS and its D3 domain are essential for an efficient HS response. The main binding site of PfAP2-HS across the full genome, mapped using ChIP-seq, coincides with the position of a tandem G-box motif in the *hsp70-1* promoter. Weaker binding (not significant in some replicate experiments) was observed at the *hsp90* promoter, also at the position of a tandem G-box, whereas no binding was observed in the PF3D7_1421800 upstream region that lacks a G-box. Therefore, the combination of transcriptomic and ChIP-seq data restricts the direct targets of PfAP2-HS during HS to *hsp70-1* and possibly *hsp90*. Since this is consistent with the *in vitro* specificity of recombinant D1 [69], it is likely that PfAP2-HS binds the promoter of its target genes via D1, and D3 participates in other interactions (e.g. protein-

297 protein interactions) necessary for transcriptional activation during HS. Nothing
298 is known about the function of D2.
299
300 The characterization of transcriptional changes after exposure to HS in wild type
301 parasites compared to parasites deficient for PfAP2-HS provided the
302 opportunity to disentangle the PfAP2-HS-dependent HS response from other
303 transcriptional changes occurring during HS. The HS response was studied at
304 the mature trophozoite stage, which is the asexual blood stage most sensitive to
305 HS [43]. Genes that are activated during HS in wild type parasites but not in
306 PfAP2-HS mutants can be confidently ascribed to the protective HS response:
307 parasites that activate these genes survive HS, whereas parasites that fail to do
308 so are dramatically more sensitive to HS. Therefore, *hsp70-1*, *hsp90* and
309 PF3D7_1421800 constitute the PfAP2-HS-dependent *P. falciparum* protective
310 HS response. This is a canonical response that is triggered by an external
311 condition (elevated temperature), like a frilled lizard spreading out the frill when
312 threatened.
313
314 Classifying all the other genes that are altered during HS is less straight
315 forward. It can be reasoned that transcript level changes that reflect cell
316 damage or death are expected to be more pronounced in PfAP2-HS-deficient
317 lines than in wild type parasites, because the former lines suffer increased cell
318 damage during HS, as reflected by their lower survival (**Figure 3**). Hundreds of
319 genes showed this pattern [43]. These changes are like the wrinkled skin of a
320 starving cow, which is more pronounced when the damage is more severe: a
321 marker of damage, not a protective response. However, the activation of a

putative PfAP2-HS-independent HS response may also be more pronounced in PfAP2-HS-deficient parasites, as the higher cell damage may trigger a stronger response. Therefore, it is impossible to distinguish which transcript level changes that are more pronounced in the mutant lines than in wild-type parasites reflect cell damage and which are part of a potentially broad PfAP2-HS-independent HS response (**Figure 3**). In any case, what is clear is that PfAP2-HS-dependent gene activation is essential for HS survival, as the putative PfAP2-HS independent response alone is unable to secure parasite survival under thermal stress.

HSP70-1 was shown to be essential for HS survival using a conditional knockdown approach [71]. Several other *P. falciparum* proteins, including RESA, GRP170, HSP70-x and HSP110, were also shown to be essential for HS survival using knockout or knockdown approaches, but the expression of the genes encoding these proteins is not activated or repressed during HS [67,72-74]. Stabilization of the digestive vacuole by the lipid phosphatidylinositol 3-phosphate (PI(3)P) [71], Pfj4 [75], PfGCN5 [45], synthesis of isopentenyl pyrophosphate and farnesylation of cytosolic type I HSP40 [76,77] also play important roles in HS survival. Furthermore, a recent large-scale forward genetics phenotypic screen identified more than a hundred genes in which transposon insertion in the coding or neighbor intergenic regions was associated with increased HS sensitivity, indicating that these genes are also needed for HS survival [44]. Two of these genes were proposed to contribute to the regulation of the HS response. However, the expression of many of the genes needed for HS survival identified in this study did not change during HS.

Together, these studies revealed that there are many genes that are essential for HS survival but their basal expression levels are sufficient for their role during HS. Therefore, notwithstanding their importance for HS survival, these genes are not part of a response (**Figure 4**). They are like the skin of the rhino, that is permanently present regardless of the conditions of the environment.

The master regulators of the protective HS response in evolution: prokaryotes, eukaryotes… and *Plasmodium*

In prokaryotes, the best characterized master regulator of the HS response is the *E. coli* σ^{32} protein. In eukaryotes, until the identification of PfAP2-HS, the **heat shock factor 1 (HSF1)** was considered the universal master regulator of the HS response. The remarkable conservation of HSF1 and its cognate DNA binding site in most eukaryotes, from yeast to humans, suggests that they already existed in the last eukaryotic common ancestor [78-81]. The notable exception are most protozoan parasites, including *Plasmodium* spp., where HSF1 orthologs have not been identified.

In *E. coli*, σ^{32} binds the cognate CCCCATWT and TTGAAA motifs in the -10 and -35 regions of the target gene promoters [82,83]. The σ^{32} protein is normally repressed by chaperones and proteolytically degraded, but this repression is released when the chaperones bind unfolded proteins upon HS [83-85]. HSF1 proteins have several conserved and some species-specific domains [78,79] (**Figure 2**). Activation of HSF1 during HS involves titration of bound chaperones by unfolded proteins, similar to σ^{32}, and also multiple posttranslational modifications and trimerization, which enables binding to the

372 heat sock element (HSE) in the promoter of target genes. The HSE consists of
373 at least three inverted repeats of the nGAAn sequence [78-81].
374
375 *E. coli* σ^{32} regulates over a hundred genes during HS, of which *hsp70* and
376 *hsp90* orthologs are among the most highly upregulated genes [82]. In model
377 eukaryotes, HSF1 activates a compact set of typically <100 genes that also
378 include *hsp70* and *hsp90* genes and other genes linked to proteostasis [86-90].
379 Remarkably, the fundamental characteristics of the HS response, such as rapid
380 and transient activation of key chaperone-encoding genes that minimally
381 include *hsp70* and *hsp90* **(Box 4)**, are conserved even across different domains
382 of life (prokaryotes and eukaryotes), and also in *P. falciparum*. While σ^{32}, HSF1
383 and PfAP2-HS do not share any recognizable sequence or structural similitude
384 (**Figure 2**), they play an analogous role driving a similar response. This is
385 suggestive of convergent evolution between the transcription factors that
386 orchestrate the protective HS response in bacteria, most eukaryotes and
387 *Plasmodium*.
388
389 HSF1 and PfAP2-HS share additional features. Both the HSF1- and PfAP2-HS-
390 dependent responses are essential for HS survival, but of the hundreds of
391 genes that change expression during HS (both in model eukaryotes and in *P.*
392 *falciparum*), only a small subset of genes linked to proteostasis show rapid,
393 transient activation regulated by HSF1 or PfAP2-HS [43,78,87,89]. The majority
394 of genes that show increased expression during HS in model eukaryotes are
395 regulated by other transcription factors that drive a more general stress
396 response [81,89], such as the serum response factor (SRF) in mammals [86,91]

or the Msn2 and Msn4 transcription factors in yeast [87,92]. The transcription factor(s) that regulate the putative PfAP2-HS-independent HS response in *P. falciparum* have not been identified. The downregulation of hundreds of genes during HS is also independent of the master regulators of the HS response, HSF1 or PfAP2-HS [43,81,89].

At the mechanistic level, HSF1 binding requires three repeats of the nGAAn DNA sequence [78-81], whereas PfAP2-HS preferentially binds two tandem copies of the G-box motif [43,69]. The mechanism of PfAP2-HS activation is not known, but given that HSF1 activation involves trimerization to enable binding to its cognate motif, it is tempting to speculate that oligomerization of PfAP2-HS (in this case, dimerization) may play a role in its activation. A feedback loop involving repression of PfAP2-HS under basal conditions by the chaperones that it activates upon HS, analogous to the regulation of prokaryotic σ^{32} and eukaryotic HSF1 by chaperone titration, is also a plausible regulatory mechanism that deserves to be investigated.

A further similarity between PfAP2-HS and yeast HSF1 is that both play a role in proteostasis maintenance under basal (non-stress) conditions, in addition to their role activating the HS response. Remarkably, the only essential role of yeast HSF1 under basal conditions is the regulation of *hsp70* and *hsp90* [87]. In the case of PfAP2-HS, the knockout parasite line has important growth defects at 37ºC, and several observations indicate that the role of PfAP2-HS under basal conditions is related with the maintenance of proteome integrity: i) the phenotype of the knockout line is attenuated at 35ºC; ii) under basal conditions,

the expression of *hsp70-1* and *hsp90*, among a few other genes, is reduced in the knockout line; iii) the knockout line shows increased sensitivity to **artemisinin**, which produces general proteome damage, and to inhibitors of the proteasome [43]. A link between the HS response and artemisinin resistance is also supported by the results of a forward-genetics phenotypic screen in which mutants sensitive to HS tended to show also increased sensitivity to artemisinin [44]. Of note, the growth defects of *pfap2-hs* mutants at 37ºC are strain-dependent, as they were observed in some genetic backgrounds (including the reference line 3D7), but not others. In contrast, failure to build a protective HS response, HS hypersensitivity, downregulation of basal *hsp70-1* and *hsp90* levels and increased sensitivity to artemisinin associated with *pfap2-hs* deletion occurred in all genetic backgrounds tested [43]. A recent report showed that in the *P. falciparum* NF54 line PfAP2-HS can be disrupted at 37ºC and, intriguingly, this resulted in reduced gametocyte production [93].

CONCLUDING REMARKS

It is often complex to distinguish between an actual response to stress conditions and transcriptional changes that reflect cell damage produced by this condition. However, recent research has unambiguously demonstrated that malaria parasites are able to respond to changes in their environment at the transcriptional level. This has been established for some metabolic conditions and for exposure to febrile temperatures. Knowledge of the molecular sensors that detect these conditions, the signal transduction pathways and the transcriptional regulators that drive the responses is still incomplete. These

important research gaps should be the subject of future research (see **Outstanding Questions**).

The HS response provides the opportunity to study the molecular mechanisms of a directed transcriptional response in *P. falciparum*, because the transcription factor regulating the response has been identified and it drives rapid and transient upregulation of a defined set of genes directly related with protection from thermal stress. Additionally, unlike other malarial transcription factors that appear to regulate hundreds of target genes and possibly operate in a complex combinatorial manner [51,94-97], PfAP2-HS has an extremely compact set of defined target genes, which makes it a more tractable system. Furthermore, PfAP2-HS is not essential under culture conditions, which facilitates its characterization.

The basic features of the *P. falciparum* PfAP2-HS-driven HS response are similar to the HSF1-driven HS response in other eukaryotes. However, in *P. falciparum* the small size of the set of target genes is extreme, and the response depends on a transcription factor evolutionarily unrelated with the conserved transcription factor that drives the HS response in all other eukaryotes studied so far. This makes the malarial HS response intriguing and unique. Further characterization of this response is expected to reveal new peculiarities of the fascinating biology of this important human pathogen.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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GLOSSARY

Artemisinin: potent antimalarial compound derived from the plant *Artemisia annua*. It rapidly kills asexual blood-stage *Plasmodium* spp. parasites and has partial activity against gametocytes. Artemisinin derivatives are the key component of the current frontline treatment against malaria.

Bet-hedging: adaptive strategy based on phenotypic heterogeneity among the individuals of a population to enable survival of the population when the environment changes. This strategy reduces population fitness variance (across environments) at the cost of reducing mean fitness.

Chaperone (or molecular chaperone): protein that assists folding and assembly of other macromolecules (typically proteins), contributes to their stability or prevents their aggregation without being present in their final structure. Heat shock proteins (HSPs) are a type of chaperones that show increased expression upon HS.

Directed transcriptional response: reaction of an organism to a change in its environment, involving changes in gene expression. These changes are typically driven by transcription factors and affect genes that favor survival under the new conditions of the environment.

Heat shock (HS): temperature increase that can trigger the HS response. In *P. falciparum* studies, HS assays are typically used to mimic malarial fever episodes.

Heat shock factor 1 (HSF1): eukaryotic transcription factor, conserved from yeast to humans, that binds the conserved heat shock element (HSE) DNA motif and upon HS rapidly drives a massive and transient increase in the

expression of a relatively small set of genes mainly involved in proteostasis maintenance.

Heat shock response (HS response): transcriptional changes that ensue after exposure of an organism to temperatures above its optimal growth temperature, aimed at protecting the cell from proteome damage and other heat-induced damage. In model eukaryotes, the HS response is often defined as only the HSF1-dependent changes.

Intraerythrocytic development cycle (IDC): growth cycle that starts when a malaria parasite at the merozoite stage invades an erythrocyte and develops through the ring, trophozoite and multinucleated schizont stages. Next, the schizont bursts and releases new merozoites. In *P. falciparum,* the IDC lasts ~48 h and is responsible for all malaria symptoms.

***Plasmodium falciparum*:** *Plasmodium* spp. are protozoan parasites from the phylum Apicomplexa that cause malaria disease. Of the five species that cause malaria in humans, *P. falciparum* is the deadliest.

Proteostasis (or protein homeostasis): refers to the basal state in which protein levels, folding, interactions and localization within a cell are correctly maintained. It involves a network of protein quality control pathways that regulate from protein synthesis to stability and degradation.

Box 1. The ApiAP2 family of transcription factors.

The Apicomplexan AP2 (ApiAP2) family is the largest family of transcription factors identified in apicomplexan parasites [53]. In *P. falciparum*, it comprises 27 proteins with one to three apetala2 (AP2) DNA-binding domains [51,98].

The AP2 domain, of around 60 amino acids, was originally identified in transcriptional regulators from plants that participate mainly in development and environmental stress responses. In *P. falciparum*, ApiAP2 proteins include transcriptional activators and repressors, and are considered the main regulators of life cycle progression. Specific members of the ApiAP2 family play important roles at different stages of the developmental cycle, including blood stages, liver stages and mosquito stages (reviewed in [51]). Systematic knockout screens targeting the entire ApiAP2 family in rodent malaria parasites [99,100] and more recently in *P. falciparum* [93] revealed that many of the genes were refractory to disruption, suggesting that they are essential for asexual blood growth. In addition to controlling the transcriptional program at each stage of development, other roles have been proposed for specific ApiAP2 proteins, including a developmental decision, telomere maintenance, heterochromatin biology or driving a transcriptional response [43,51,93,101]. However, the function of several ApiAP2 proteins still remains unknown.

The cognate DNA motif recognized by the majority of individual *P. falciparum* AP2 domains has been identified *in vitro* using recombinant AP2 domains [69], and the genome-wide occupancy of some of the *P. falciparum* ApiAP2 proteins has been mapped using ChIP-seq [43,51,93,94,96,101,102].

838

839 **Box 2. How is malaria fever triggered?**

840 Malaria fever is triggered by schizont rupture at the end of each round of the
841 IDC, when new merozoites and parasite-derived molecules are released into
842 the bloodstream. Several parasite-derived molecules have been proposed to
843 induce proinflammatory host responses that lead to fever, but the relative
844 importance of each of these molecules in triggering fever is not completely
845 understood. These parasite molecules are often referred to as malaria toxins
846 [65,103,104].

847

848 A main parasite toxin proposed to induce fever is glycosylphosphatidylinositol
849 (GPI), an abundant glycolipid that mediates membrane anchoring of many
850 parasite proteins and is also found as a free molecule [65,103-105]. Another
851 abundant parasite-derived toxin is the malarial pigment, called hemozoin, which
852 results from crystallization by the parasite of toxic free heme derived from
853 hemoglobin digestion. Hemozoin has been proposed to trigger malaria fever,
854 either by itself or acting as a carrier to present other attached molecules such
855 as parasite DNA [103,106]. Uric acid and parasite RNA can also induce a
856 proinflammatory response leading to fever [103,107,108].

857

858 These toxins are recognized by specific toll-like receptors (TLR) [103,104,106],
859 which start a signaling cascade that involves tumor necrosis factor (TNF) and
860 other pyrogenic cytokines (e.g., IL-6 and IL-1) and secretion of prostaglandin E2
861 (PGE2) [103,107,109]. This acts on warm-sensitive neurons in the preoptic area
862 of the hypothalamus, which trigger effector responses such as shivering,

863 thermogenesis and peripheral vasoconstriction that ultimately result in fever
864 [110]. While TNF appears to play a role as a mediator of malaria fever, the
865 capacity of parasite-derived toxins to rapidly induce production of TNF [65] was
866 put into question when it was realized that some of the early studies that
867 reported massive stimulation of TNF production by parasite lysates used *P.*
868 *falciparum* cultures contaminated with *Mycoplasma*, a potent stimulator of
869 macrophages [111].
870
871 **Box 3. Patterns of malaria fevers.**
872 Periodical fever is the hallmark of clinical malaria. In *P. falciparum* infections,
873 fever paroxysms follow a regular sequence of events that begins with chills,
874 followed by the rise in temperature and rigors; afterwards, the temperature falls
875 slowly, finishing with severe sweating [109]. The periodicity of fever peaks,
876 which are triggered by schizont rupture, in principle coincides with the duration
877 of the IDC of the different *Plasmodium* species: 48 h (tertian fever) in *P.*
878 *falciparum*, *P. vivax* and *P. ovale* infections, and 72 h (quartan fever) in *P.*
879 *malariae*. However, the actual patterns observed in many patients are far from
880 these canonical regular patterns, especially in *P. falciparum* infections, in which
881 fever peaks can occur daily (quotidian fever) or every 36 h (subtertian fever)
882 [47,56,61,62,109]. Fever patterns often vary during the course of an infection,
883 and erratic patterns appear to be associated with high parasite multiplication
884 rates and host anti-malarial immunity [47,65,112].
885
886 The most likely explanation for the irregular fever patterns is lack of
887 synchronicity of the parasite population (i.e., schizont rupture does not occur

simultaneously within a short time window every 48 h) [62], either by the presence of multiple infections with non-synchronized phases or by non-synchronous single infections. An important implication of the commonly observed irregular fever patterns is that in human malaria infections parasites at all stages of the IDC, rather than only bursting schizonts and newly formed rings, are exposed to febrile temperatures.

The duration and severity of fever episodes in malaria infections also shows a high level of variability. The length of the entire fever episode (>37ºC) can vary from 7 to 24 h, with a peak at >38.5ºC that can last between 2.5 and 10 h and in some cases spikes at >40ºC that typically last about 1 h [47,56,61,62,109,112].

Box 4. Heat shock proteins.

Heat shock proteins (HSPs) are molecular chaperones that are induced at above-optimal temperatures as part of the HS response. HSPs assist protein refolding and prevent nonspecific protein aggregation in the cytoplasm during thermal stress and other types of stress, contributing to reestablish proteostasis. However, they also play essential roles in *de novo* protein folding and stabilize the proteome under non-stress conditions. HSPs generally bind unfolded proteins promiscuously by recognizing stretches of exposed hydrophobic amino acids. Chaperone binding prevents non-specific aggregation, which can be lethal to the cell, and actively promotes folding in a process that uses ATP. Since chaperones bind unfolded client proteins in stoichiometric ratios, they are required in large amounts and are among the most abundant proteins in the cytoplasm [66,68].

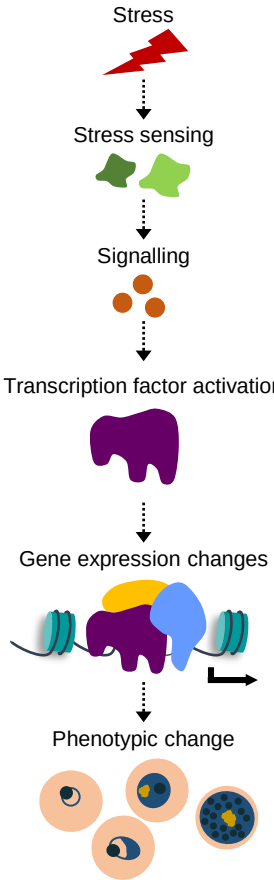
913

914 Several chaperone classes, highly conserved in prokaryotes and eukaryotes,
915 have been described. The HSP70 system involves the HSP70 protein, one of
916 the most conserved chaperones, and co-factors of the HSP40 and other
917 families. HSP70 uses binding and release cycles to prevent protein
918 aggregation, to facilitate spontaneous correct folding and even to refold
919 aggregated proteins. The HSP70 system appears to operate on the majority of
920 proteins, whereas chaperonins and the HSP90 system operate downstream on
921 specific proteins that cannot be correctly folded by the HSP70 system alone. In
922 eukaryotes, most chaperonins are not induced by heat shock, are relatively
923 slow and their role is restricted to a relatively small number of client proteins. In
924 contrast, the sophisticated HSP90 system is important for the folding of proteins
925 involved in many pathways, although it is less promiscuous than the HSP70
926 system. Other conserved chaperone classes are the HSP100 family, which are
927 not present in the cytoplasm of higher eukaryotes, and the ATP-independent
928 small HSPs, which bind partially folded proteins to passively prevent
929 aggregation, but do not contribute to protein folding [66,68,113,114].

930

931 The genome of *P. falciparum* encodes all major classes of eukaryotic
932 chaperones, including six chaperones of the HSP70 family and four of the
933 HSP90 family [115]. The canonical cytosolic chaperones of these families,
934 which are highly abundant, are HSP70-1 (PF3D7_0818900) and HSP90
935 (PF3D7_0708400), respectively [116]. The reason why in *P. falciparum* the
936 genes that encode these two chaperones are rapidly and transiently
937 upregulated during HS, whereas other HSPs are either not upregulated or are

938 upregulated later by other transcription factors, may be related with the central
939 role of the HSP70 and HSP90 systems in general proteostasis maintenance.
940
941



	Calorie restriction	LysoPC depletion		Heat shock
Stress	High AMP/ATP ratio	Low levels of precursors for phospholipid synthesis		Cytoplasmic proteotoxic stress
Stress sensing	KIN kinase	?		?
Signalling		?	GDV1	?
Transcription factor activation	?	?	AP2-G	AP2-HS
Gene expression changes	Multiple genes	*ek* *pmt*	Early gametocyte markers	*hsp70-1* *hsp90*
Phenotypic change	Attenuated replication and virulence	Metabolic rewiring	Sexual conversion	Protein homeostasis

Figure 1. Examples of directed transcriptional responses in malaria parasites. The schematic at the left indicates the steps of a canonical directed transcriptional response. The key factors identified for each step in three clear examples of *Plasmodium* spp. directed transcriptional responses are indicated. Only some key genes that change expression as part of the response are listed. A question mark (?) indicates that the factor(s) involved have not been identified.

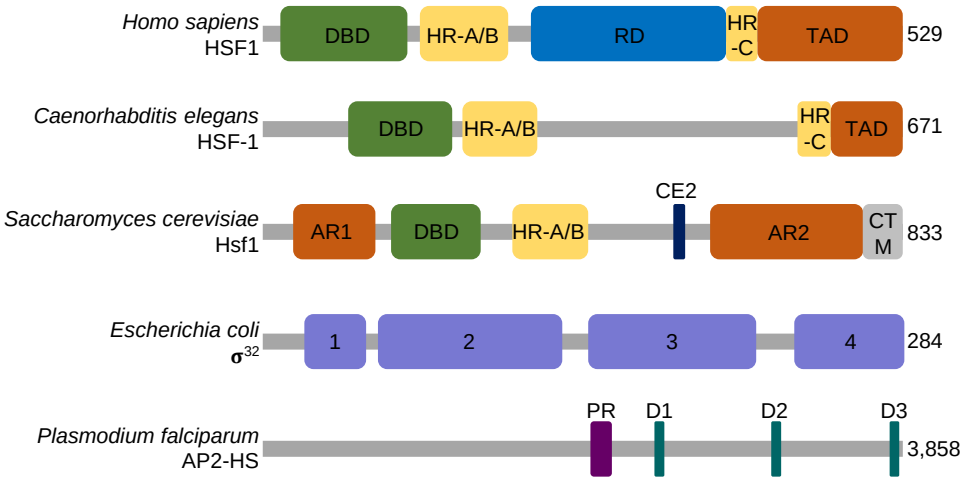

Homo sapiens
HSF1
DBD
HR-A/B
RD
HR
-C
TAD
529
Caenorhabditis elegans
HSF-1
DBD
HR-A/B
HR
-C
TAD
671
Saccharomyces cerevisiae
Hsf1
AR1
DBD
HR-A/B
CE2
AR2
CT
M
833
Escherichia coli
σ32
1
2
3
4
284
Plasmodium falciparum
AP2-HS
PR
D1
D2
D3
3,858

Figure 2. Recognizable domains in the transcriptional regulators that orchestrate the protective HS response in different organisms. The number of amino acids of each protein is specified on the right (schematic not to scale between the different proteins). DBD, DNA-binding domain; HR-A/B and -C, heptapeptide repeat A, B or C; RD, regulatory domain; TAD, transcriptional activation domain; AR1 and AR2, transcriptional activation regions 1 and 2; CE2, conserved element 2; CTM, C-terminal modulator domain; 1, 2, 3 and 4, σ^{32} domains 1, 2, 3 and 4; PR, Pentapeptide-repeat-like; D1, D2 and D3, apetala2 (AP2) domains 1, 2 and 3.

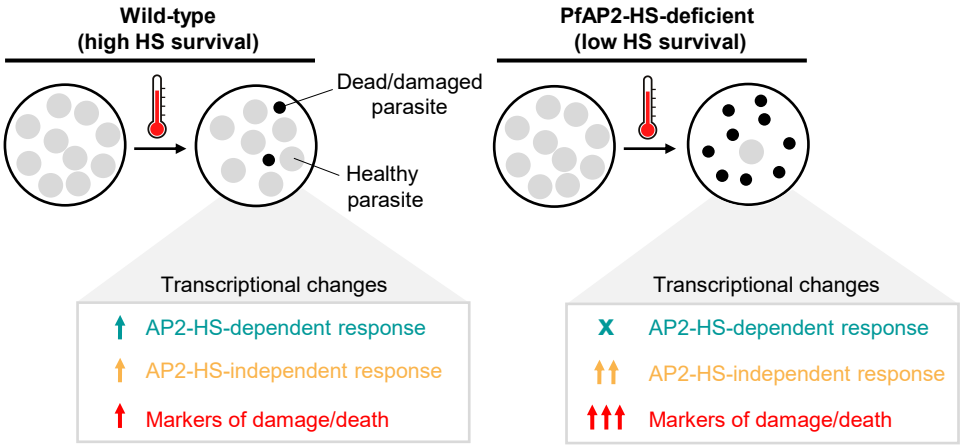

Wild-type
(high HS survival)
Dead/damaged
parasite
Healthy
parasite
Transcriptional changes
AP2-HS-dependent response
AP2-HS-independent response
Markers of damage/death
PfAP2-HS-deficient
(low HS survival)
Transcriptional changes
X
AP2-HS-dependent response
AP2-HS-independent response
Markers of damage/death

Figure 3. Transcriptional changes upon HS in wild-type parasites and in parasites deficient for PfAP2-HS. Transcriptional changes upon HS that depend on PfAP2-HS do not occur in parasites deficient for this transcription factor. Since the PfAP2-HS-driven response protects the cell during heat stress, parasites lacking PfAP2-HS suffer increased cell damage and death. Therefore, alterations that reflect cell damage or death are increased in the PfAP2-HS-deficient parasites. Putative changes in gene expression that are part of a protective response but are regulated by different transcription factor(s) (PfAP2-HS-independent response) are also increased in the deficient parasites, as the increased cell damage triggers a stronger response. A thermometer indicates exposure to febrile temperatures.

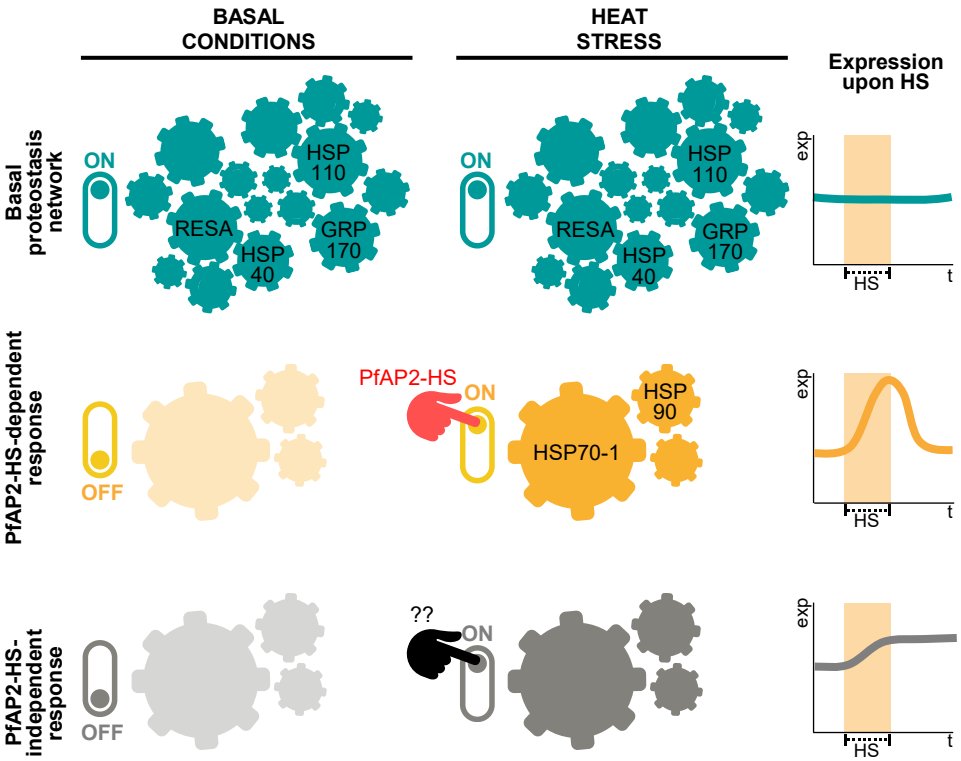

BASAL CONDITIONS
HEAT STRESS
Expression upon HS
Basal proteostasis network
ON
HSP 110
RESA
GRP 170
HSP 40
ON
HSP 110
RESA
GRP 170
HSP 40
exp
HS
t
PfAP2-HS-dependent response
OFF
PfAP2-HS
ON
HSP 90
HSP70-1
exp
HS
t
PfAP2-HS-independent response
OFF
??
ON
exp
HS
t

Figure 4. Schematic of the different mechanisms that contribute to proteostasis maintenance during HS in *P. falciparum*. A large number of proteins necessary for proteostasis maintenance during HS are constitutively expressed (top, basal proteostasis network): the transcript levels of the genes encoding these proteins are similar under basal (non-stress) or heat stress conditions. These proteins are not part of a response. In contrast, the expression of the genes encoding other proteins is increased in reaction to thermal stress. The activation of some of these genes depends on PfAP2-HS (middle, PfAP2-HS-dependent HS response), whereas the activation of other genes does not (bottom, PfAP2-HS-independent HS response). The transcription factor(s) that regulate the putative PfAP2-HS-independent response have not been identified. Examples of proteins in the different categories are shown except for the PfAP2-HS-independent response, because proteins in this category cannot be unambiguously distinguished from markers of cell damage. The schematic plots at the right represent the typical expression patterns for the genes encoding the proteins in the different categories during and after HS, with unaltered transcript levels for the basal proteostasis network, a rapid and transient increase in expression for the PfAP2-HS-dependent response, and a slower but more sustained increase for the PfAP2-HS-independent response (t: time; exp: expression levels; the area shaded in yellow indicates the period in which parasites are exposed to HS).