The PfAP2-HS transcription factor protects malaria parasites from febrile temperatures

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24 **Periodic fever is the most characteristic clinical feature of human malaria¹⁻**
 25 **³, but how parasites survive febrile episodes is not known. While**
 26 ***Plasmodium* spp. genomes encode a full complement of chaperones⁴,**
 27 **they lack an ortholog of the conserved transcription factor HSF1, which in**
 28 **most eukaryotes activates the expression of key chaperones upon heat**
 29 **shock (HS)⁵⁻⁸. Here we identified PfAP2-HS, a transcription factor of the**
 30 **ApiAP2 family⁹⁻¹¹, as the key regulator of the *P. falciparum* protective HS**
 31 **response. The PfAP2-HS-dependent HS response is largely restricted to**
 32 **rapid activation of *hsp70-1*, the predominant direct target of PfAP2-HS,**
 33 **and *hsp90*. Deletion of PfAP2-HS dramatically reduced HS survival and**
 34 **also resulted in severe growth defects at 37°C, but not at 35°C, and**
 35 **increased sensitivity to imbalances in protein homeostasis (proteostasis)**
 36 **produced by artemisinin, the current frontline antimalarial drug^{12,13}. These**
 37 **results demonstrate that PfAP2-HS contributes to general maintenance of**
 38 **proteostasis and drives a rapid chaperone-based protective response**
 39 **against febrile temperatures. While several ApiAP2 transcription factors**
 40 **regulate life cycle transitions in malaria parasites^{11,14,15}, PfAP2-HS is the**
 41 **first identified *Plasmodium* transcription factor that controls a protective**
 42 **response to a within-host environmental challenge.**

43

44 A temperature increase of just a few degrees above the optimal growth
 45 temperature can cause aberrant protein folding and aggregation, resulting in a
 46 proteostasis imbalance that can lead to cell-cycle arrest or even cell death¹⁶. To
 47 counteract the effect of high temperatures and other proteotoxic conditions,
 48 living organisms use a complex array of molecular chaperones that assist

49 protein refolding and prevent non-specific aggregation^{16,17}. This is especially
 50 important for human malaria parasites, which are exposed to periodic fever
 51 episodes during blood stages. In *P. falciparum* infections, which are responsible
 52 for the most severe forms of human malaria, fever typically occurs on alternate
 53 days (tertian fever). This reflects the ~48 h duration of the asexual
 54 intraerythrocytic development cycle (IDC), during which parasites progress
 55 through the ring, trophozoite and multinucleated schizont stages. Schizont
 56 rupture and release of new invasive merozoites trigger fever episodes, an
 57 important component of the innate immune response that limits the total
 58 parasite burden^{2,3,18}.

59
 60 To understand the molecular basis of HS resistance in *P. falciparum*, we first
 61 analysed parasite lines that we had previously selected with periodic HS over
 62 five consecutive rounds of the IDC¹⁹ (Supplementary Fig. 1a). Although the
 63 parental parasite line (3D7-A) appeared to have lost the ability to withstand
 64 febrile temperatures during growth in culture, its ability to quickly re-adapt to HS
 65 pressure suggested that this line contained a selectable subpopulation of
 66 parasites resistant to HS, which could have either a genetic or an epigenetic
 67 basis. Transcriptomic analysis across the full IDC under basal conditions (no
 68 HS) of two independently selected lines (3D7-A-HS r1 and r2) and non-selected
 69 cultures maintained in parallel (3D7-A r1 and r2) failed to identify any basal
 70 transcript level differences that could explain the HS resistance phenotypes
 71 (Supplementary Fig. 1b-d). Therefore, we sequenced the genomes of these
 72 lines, which revealed a novel single nucleotide polymorphism (SNP) that was
 73 predominant in non-selected cultures, but virtually absent after HS selection

(Fig. 1a-b, Supplementary Table 1). The mutation is also absent from two other 3D7 stocks in our laboratory and from the 3D7 reference genome, indicating that it arose spontaneously in the 3D7-A stock during culture. This SNP results in a premature STOP codon (Q3417X) in the gene PF3D7_1342900, which encodes a putative transcription factor of the ApiAP2 family⁹⁻¹¹ that we termed PfAP2-HS. PfAP2-HS has three AP2 domains (D1-D3), and the Q3417X mutation results in a truncated protein that lacks D3 (PfAP2-HSΔD3) (Fig. 1c). Therefore, adaptation to HS involved elimination of parasites that expressed truncated PfAP2-HS, consistent with a role for this protein in the HS response. In support of this idea, the first AP2 domain (D1) of PfAP2-HS was previously reported to recognize *in vitro* a DNA motif termed G-box¹⁰, which is enriched in the upstream region of some HS protein (HSP) chaperone genes²⁰.

To test the involvement of PfAP2-HS in HS resistance, we used a HS survival assay with a 3 h HS at 41.5°C¹⁹ at the mature trophozoite stage, because maximal survival differences between HS sensitive and resistant parasite lines were observed when exposing parasites at this stage (Supplementary Fig. 1e). The analysis of a collection of 3D7-A subclones revealed that all subclones with the Q3417X mutation show a HS-sensitive phenotype, whereas subclones with the wild-type allele show a resistant phenotype (Fig. 1d).

To further characterize PfAP2-HS, we disrupted the entire gene using CRISPR-Cas9 technology. After several unsuccessful attempts with different 3D7 subclones at 37°C (the physiological temperature for *P. falciparum*), we reasoned that PfAP2-HS may play a role in regulating the expression of

99 chaperones under basal conditions, in addition to being necessary for HS
100 survival. Therefore, we attempted to knock out the gene in cultures maintained
101 at 35°C, as mild hypothermia is expected to reduce protein unfolding and favour
102 proteome integrity^{21,22}. Indeed, at 35°C, knockout of *pfap2-hs* was readily
103 achieved in both the HS-resistant 10E and the HS-sensitive 10G subclones of
104 3D7-A (10E_Δ*pfap2-hs* and 10G_Δ*pfap2-hs* lines) (Fig. 1c, Supplementary Fig.
105 2a). Deletion of *pfap2-hs* resulted in severely increased sensitivity to HS, with a
106 level of HS survival below that of parasites expressing PfAP2-HSΔD3. Deletion
107 of the gene in two parasite lines of unrelated genetic background, HB3 and
108 D10, also resulted in a major reduction in HS survival (Fig. 1e).

109

110 To define the PfAP2-HS-dependent and independent HS response, we
111 conducted a transcriptomic time-course analysis of the 10E (wild-type PfAP2-
112 HS), 10G (PfAP2-HSΔD3) and 10E_Δ*pfap2-hs* lines during and after HS (Fig.
113 2a, Supplementary Fig. 3a, Supplementary Table 2). Hierarchical clustering
114 based on changes relative to cultures not exposed to HS revealed only three
115 genes in the full genome with a rapid increase in transcript levels upon HS that
116 occurred in 10E but not in 10G or 10E_Δ*pfap2-hs* (cluster I). This cluster
117 comprises a gene of unknown function (PF3D7_1421800), the cytoplasmic
118 *hsp70* (*hsp70-1*; PF3D7_0818900) and *hsp90* (PF3D7_0708400) (Fig. 2a).
119 These two chaperone-encoding genes contain in their regulatory regions the
120 best two matches in the full genome for a tandem G-box^{10,20} (Supplementary
121 Fig. 3b), suggesting an important role for this tandem arrangement. The
122 strongest transcriptional response was observed for *hsp70-1* (~16-fold increase
123 versus ~4-fold for *hsp90*). To validate the observation that rapid activation of

124 cluster I genes upon HS depends on PfAP2-HS and requires its D3, we used
 125 *pfap2-hs* knockout parasite lines of different genetic backgrounds and several
 126 3D7-A mutant subclones expressing PfAP2-HS Δ D3 (Supplementary Fig. 4). In
 127 all knockout and mutant lines examined, the *hsp70-1* response to HS was
 128 delayed and of much lower magnitude. These experiments also confirmed that
 129 the *hsp90* response is weaker than the *hsp70-1* response, and is delayed in
 130 PfAP2-HS mutants.

131
 132 Genes in other clusters (II-VI) of the transcriptomic analysis showed changes in
 133 expression during HS that did not depend on PfAP2-HS, as they occurred even
 134 in the knockout line (Fig. 2a). Indeed, a larger number of genes with altered
 135 transcript levels was identified in 10G and 10E_*Δpfap2-hs* than in 10E (Fig. 2b).
 136 Furthermore, the alterations persisted 2 h after HS in both HS-sensitive lines,
 137 whereas in 10E the majority of genes returned to basal expression levels (Fig.
 138 2a). This indicates that many of these alterations reflect unresolved cell damage
 139 or death, and in 10E the rapid PfAP2-HS-dependent response limited cell
 140 damage and the subsequent changes in the expression of clusters II-VI genes.
 141 Indeed, after HS the transcriptome of the 10G and 10E_*Δpfap2-hs* lines showed
 142 a more pronounced deviation from a reference transcriptome²³ than 10E (Fig.
 143 2c). Global transcriptional analysis also revealed that HS resulted in delayed
 144 IDC progression, again more pronounced in 10G and 10E_*Δpfap2-hs* (Fig. 2d).

145
 146 In addition to genes reflecting cell damage, clusters II-VI likely include some
 147 genes that participate in the PfAP2-HS-independent HS response. In particular,
 148 clusters V-VI include several chaperone-encoding genes upregulated during

149 HS, although at a later time point than cluster I genes (Fig. 2a). However, the
 150 expression of the majority of known *P. falciparum* chaperones⁴ was not altered
 151 by HS and, except for cluster I genes, the alterations occurred mainly in the
 152 mutant lines (Supplementary Fig. 5). To provide a clearer view of the wild-type
 153 HS response, we analysed changes upon HS in the 10E line alone. Overall,
 154 there was generally good concordance with the genes and processes altered
 155 upon HS described in a previous study using non-synchronized cultures²⁴
 156 (Supplementary Fig. 6, Supplementary Table 3). Altogether, we conclude that a
 157 number of genes are up or downregulated during HS and some may contribute
 158 to HS protection through PfAP2-HS-independent responses, but in the absence
 159 of the rapid PfAP2-HS-dependent activation of cluster I genes these responses
 160 are not sufficient to ensure parasite survival under febrile temperatures.

161

162 To determine the genome-wide occupancy of PfAP2-HS, we analysed a
 163 parasite line expressing endogenous HA-tagged PfAP2-HS (Supplementary
 164 Fig. 2d) using chromatin immunoprecipitation followed by sequencing (ChIP-
 165 seq). The predominant binding site of PfAP2-HS coincides with the position of
 166 the tandem G-box in the upstream region of *hsp70-1* (Fig. 2e, Supplementary
 167 Fig. 7, Supplementary Table 4). This is the only binding site with a median fold-
 168 enrichment >10 (ChIP versus input) that was consistently detected across five
 169 independent replicates, revealing an extremely restricted distribution of PfAP2-
 170 HS binding. Similar enrichment was observed between control and HS
 171 conditions by both ChIP-seq and ChIP-qPCR (Supplementary Fig. 7a,c),
 172 indicating that PfAP2-HS binds to this site constitutively and is activated *in situ*
 173 upon HS. This is similar to human HSF1, which in the absence of HS is bound

174 to its target promoters in a paused state to ensure a rapid response⁶.

175 Enrichment at the *hsp90* promoter also coincided with the position of the G-box

176 but was much weaker and, in several replicates, a significant peak was not

177 called. No enrichment was observed at the promoter of cluster I gene

178 PF3D7_1421800 that lacks a G-box motif. The only other site consistently

179 enriched for PfAP2-HS binding, albeit at much lower levels than *hsp70-1*, was

180 the upstream region of the small nucleolar RNA *snoR04* (PF3D7_0510900)

181 (Supplementary Fig. 7), which was not upregulated during HS.

182

183 Both $\Delta pfap2$ -*hs* lines of 3D7 origin (10E_ $\Delta pfap2$ -*hs* and 10G_ $\Delta pfap2$ -*hs*)

184 showed severe temperature-dependent growth defects in the absence of HS.

185 They grew at similar rates to the parental lines at 35°C, but their growth was

186 markedly reduced at 37°C or 37.5°C (Fig. 3a). The D10 $\Delta pfap2$ -*hs* line also had

187 clearly reduced growth at 37°C compared to 35°C, whereas the HB3 $\Delta pfap2$ -*hs*

188 line did not (Supplementary Fig. 8a). Both 3D7 knockout lines also showed a

189 reduced number of merozoites per schizont, especially at 37°C or 37.5°C (Fig.

190 3b), which partly explains the reduced growth rate. Additionally, even at 35°C,

191 the duration of the IDC was ~4 h longer in both knockout lines (Fig. 3c), which is

192 reminiscent of the slower life cycle progression observed in parasites under

193 proteotoxic stress²⁵. In contrast, no growth rate or life cycle duration differences

194 were observed between the 10G (PfAP2-HS Δ D3) and 10E lines, indicating that

195 D3 is necessary for HS survival but not for growth under nonstress conditions

196 (Fig. 3a-c). Normal growth at 37°C but low HS survival was also observed in

197 transgenic lines in which bulky C-terminal tags were added to the C-terminus of

198 endogenous PfAP2-HS, suggesting interference of the tag with the function of

199 D3, located only 18 amino acids from the end of the protein (Supplementary
200 Fig. 2).

201

202 Genome-wide sequence analysis has recently revealed that non-sense
203 mutations commonly emerge in *pfap2-hs* during the adaptation to culture
204 conditions^{26,27}, whereas they are virtually absent from clinical isolates (in the
205 www.malariagen.net/data/pf3k-5 dataset²⁸, only one out of >2,500 isolates
206 carries a high-confidence SNP resulting in a premature stop codon). This
207 indicates a likely essential role for PfAP2-HS in human infections, and relaxed
208 selection against loss-of-function when parasites are not exposed to febrile
209 conditions. We chose for further analysis a culture-adapted isolate carrying a
210 mutation that results in truncation of PfAP2-HS before its first AP2 domain²⁶
211 (monoclonal Gambian Line 1, PfAP2-HS_ΔD1-3), and found that a single HS
212 resulted in strong selection against parasites carrying the mutation. In contrast,
213 there was relatively weak selection against mutants during culture either at
214 35°C or 37°C, as the prevalence of the mutation only decreased from ~50% to
215 ~20% after culturing for 23 generations (Supplementary Fig. 8b-d). Consistent
216 with these results, a subclone carrying the mutation (1H) was more sensitive to
217 HS than a wild-type subclone (4E), but both showed no measurable difference
218 between growth at 35°C or 37°C (Supplementary Fig. 8e-g). Together, these
219 results indicate that PfAP2-HS is essential for HS survival in all the genetic
220 backgrounds tested. However, PfAP2-HS is necessary for normal progression
221 through the IDC at 37°C only in some genetic backgrounds (i.e., 3D7 and D10),
222 whereas in others (HB3 and the Gambian isolate) it is not essential.

223

224 To gain insight on the molecular basis of the growth defects of some of the
 225 knockout lines, we compared the trophozoite transcriptome of 10E_*Δpfap2-hs*
 226 with that of the parental 10E under basal (no HS) conditions. This revealed only
 227 a small set of genes with a ≥ 2 fold-decrease in transcript levels, which included
 228 *hsp70-1*, the direct PfAP2-HS target *snoR04* RNA and several genes mainly
 229 involved in ribosome formation. Transcript levels for *hsp90* were also reduced
 230 (< 2 fold-decrease) in the knockout line (Supplementary Fig. 9a-b). Lower
 231 transcript levels of *hsp70-1* and *hsp90* under basal conditions in 10E_*Δpfap2-hs*
 232 mature trophozoites were independently confirmed by RT-qPCR, and also
 233 observed at the late ring stage and in the knockout lines of D10 and HB3
 234 genetic background (Supplementary Fig. 9c). These results indicate that PfAP2-
 235 HS contributes to regulating the basal expression of the same chaperone-
 236 encoding genes that it activates upon HS, among a few other genes. Together
 237 with the observation that the growth defect of the *pfap2-hs* knockout lines is
 238 attenuated at 35°C, this suggests that knockout parasites have reduced
 239 proteostasis capacity, such that at 37°C they are at the edge of proteostasis
 240 collapse. Parasite lines that can grow normally at 37°C in spite of PfAP2-HS
 241 deletions including the three AP2 domains therefore must have alternative
 242 pathways active to ensure basal proteostasis. We hypothesise that mutant
 243 parasites expressing truncated PfAP2-HS are frequently selected under culture
 244 conditions because the truncations don't pose a fitness cost at 37°C in the lines
 245 in which they appear, and they prevent unnecessary activation of the HS
 246 response, which can be detrimental²⁹, by unintended mild stress that may occur
 247 during culture.

248

Artemisinins are potent antimalarial drugs that kill parasites by causing general protein damage^{12,13}, whereas resistance is associated with mutations in the Kelch13 protein^{30,31} and involves cellular stress response pathways such as the ubiquitin/proteasome system and the ER-based unfolded protein response (UPR)^{12,13,32,33}. Since PfAP2-HS regulates the expression of key chaperones, we tested the sensitivity of PfAP2-HS-deficient lines to dihydroartemisinin (DHA), the active metabolite of artemisinins. In all four different genetic backgrounds (3D7, D10, HB3 and Gambian isolate), knockout of *pfap2-hs* (or truncation before D1) resulted in higher sensitivity to a pulse of DHA than in lines with full PfAP2-HS, both at the ring or the trophozoite stage, whereas 10G showed increased sensitivity only when exposed at the trophozoite stage (Fig. 3d, Supplementary Fig. 8h-i). These results indicate that deletion of PfAP2-HS renders parasites more sensitive to chemical proteotoxic stress, in addition to HS, likely as a consequence of basal defects in cellular proteostasis. We reasoned that if parasites lacking the PfAP2-HS protein bear constitutive proteome defects, they should have low tolerance to disruption of other factors involved in proteostasis maintenance. Indeed, the 10E_Δ*pfap2-hs* line was more sensitive to the proteasome inhibitor epoxomicin than the parental 10E line or the 10G line (Fig. 3e). Furthermore, after HS, there was more accumulation of polyubiquitinated proteins in the knockout line than in 10E or 10G, reflecting higher levels of unresolved protein damage (Supplementary Fig. 8j). We also assessed the links between the PfAP2-HS-driven HS response and the other main cell stress response pathway, the UPR. Using phosphorylation of eIF2α as a UPR marker, we found that the UPR does not depend on PfAP2-HS

273 and is not directly activated by HS, because the marker was significantly
 274 elevated after HS only in the knockout line (Supplementary Fig. 8k).
 275
 276 Altogether, our results demonstrate that the PfAP2-HS transcription factor binds
 277 to the tandem G-box DNA motif at the promoter of a limited number of critical
 278 chaperone-encoding genes, *hsp70-1* and *hsp90*, and in response to febrile
 279 temperatures rapidly upregulates their expression (Fig. 3f). Specific binding to
 280 the G-box is conferred by D1¹⁰, but activation of these genes during HS strictly
 281 requires D3, which is not capable of binding DNA *in vitro*¹⁰ and likely
 282 participates in protein-protein interactions or dimerization³⁴. Other components
 283 of the protein folding machinery necessary for HS survival³⁵⁻³⁷ are constitutively
 284 expressed or induced later, but the rapid PfAP2-HS-driven response is essential
 285 to avoid irreversible damage, as parasites lacking D3 or the full protein fail to
 286 survive HS. While PfAP2-HS is not evolutionarily related with HSF1^{5,8}, the
 287 conserved master regulator of the HS response from yeast to mammals, it
 288 appears to play an analogous role. HSF1 also regulates a compact
 289 transcriptional program^{6,7} that includes the *hsp70* and *hsp90* genes. In yeast,
 290 the only essential role of this transcription factor is activating these two genes⁷.
 291 In addition to driving the protective HS response, PfAP2-HS also plays a D3-
 292 independent role under nonstress conditions, which is essential for growth at
 293 37°C in some *P. falciparum* genetic backgrounds. Several lines of evidence
 294 indicate that the role of PfAP2-HS under basal conditions is related with
 295 proteostasis maintenance (Fig. 3f), as in the case of yeast HSF1⁷. Furthermore,
 296 the malarial HS response likely mediates protection against different types of
 297 proteotoxic stress, in addition to high temperature, as reported in other

organisms^{5,16}. This idea is supported by the observation that orthologs of *pfap2*-
hs are present across *Plasmodium* spp. (Supplementary Fig. 10), including
murine malaria species that do not induce fever. The identification of the PfAP2-
HS transcription factor, which drives a strong protective response to an
environmental condition, settles the controversy of whether malaria parasites
can produce directed transcriptional responses to fluctuations in their within-
host environment³⁸ and provides a much-needed system to study induced
transcriptional responses in malaria parasites.

306

307 **METHODS**

Parasite cultures. The 3D7-A stock of the clonal *P. falciparum* line 3D7³⁹, the
3D7-A subclones 10G, 1.2B, 10E, 4D, 6D, 1.2F, W4-1, W4-2, W4-4, W4-5^{40,41},
the HB3B (mosquito and chimpanzee-passaged HB3)⁴² and D10⁴³ clonal
parasite lines, and the culture-adapted Line 1 from The Gambia²⁶ have been
previously described. The HS-selected lines 3D7-A-HS r1 and r2 were derived
from 3D7-A by exposing cultures to a 3 h HS for five consecutive cycles (each
replicate, r1 and r2, is a fully independent selection from the 3D7-A stock), and
the 3D7-A r1 and r2 lines are cultures maintained in parallel at 37°C without
HS¹⁹. The generation of the new transgenic lines is described below. Parasites
were cultured in B+ erythrocytes at a 3 % haematocrit under standard culture
conditions in RPMI-based media containing Albumax II (without human serum),
in a 5% CO₂, 3% O₂, balance N₂ atmosphere (except for cultures for ChIP-seq
experiments, in which O+ erythrocytes were used). Regular synchronization
was performed using 5 % sorbitol lysis, whereas tight synchronization (1, 2 or 5
h age window) was achieved by Percoll purification followed by sorbitol

323 treatment 1, 2 or 5 h later. All cultures were regularly maintained at 37°C, with
324 the exception of the *pfap2-hs* knockout lines that were maintained at 35°C. For
325 experiments performed in parallel with the knockout lines and other parasite
326 lines, all cultures were maintained at 35 °C from at least one cycle before the
327 experiment.

328

329 **Generation of transgenic parasite lines.** Given the large size of *pfap2-hs*
330 (11,577 bp), to knock out this gene with the CRISPR/Cas9 system we used two
331 single guide RNAs (hereafter referred to as sgRNA or guide) (Supplementary
332 Fig. 2a). One guide targets a sequence near the 5' end of the gene (position
333 866-885 from the start codon) whereas the other recognizes a sequence near
334 the 3' end (positions 11,486-11,505). The 5' guide was cloned into a modified
335 pL6-*egfp* donor plasmid⁴⁴ in which the *yfcu* cassette had been removed by
336 digestion with *NotI* and *SacII*, end blunting and re-ligation. 5' and 3' homology
337 regions (HR1: positions -2 to 808 of the gene; HR2: positions +11,520 of the
338 gene to 490 bp after the STOP codon) were also cloned in this plasmid, flanking
339 the *hdhfr* expression cassette, to generate plasmid pL7-pfap2hs_KO_sgRNA5'.
340 The 3' guide was cloned into a modified version of the pDC2-Cas9-U6-hdhfr⁴⁵
341 plasmid, in which we previously removed the *hdhfr* expression cassette by
342 digesting with *NcoI* and *SacII*, end blunting and re-ligation, and replaced the
343 *BbsI* guide cloning site by a *BtgZI* site. The resulting plasmid was termed
344 pDC2_wo/hdhfr_pfap2hs_sgRNA3'. All guides were cloned using the In-Fusion
345 system (Takara) approximately as described⁴⁴, whereas homology regions
346 were PCR-amplified from genomic DNA and cloned by ligation using restriction
347 sites *SpeI* and *AflI* (HR1), and *EcoRI* and *NcoI* (HR2).

348

349 To tag PfAP2-HS with a 2xHA-ddFKBP domain tag (1.2B_ *pfap2hs*_ddFKBP
350 line) we used a single homologous recombination approach (Supplementary
351 Fig. 2b). To generate the *pfap2hs*_HA-ddFKBP plasmid, we replaced the *pfap2*-
352 *g* homology region in plasmid PfAP2-G-ddFKBP¹⁴ by a PCR-amplified fragment
353 including positions 9,551-11,574 of *pfap2-hs* in frame with the tag. The fragment
354 was cloned using restriction sites *NotI* and *XhoI*.

355

356 For constructs aimed at C-terminal tagging of *pfap2-hs* using CRISPR/Cas9
357 (10E_ *pfap2-hs*_eYFP-Cterm and 10E_ *pfap2-hs*_3xHA-Cterm lines) we used a
358 guide corresponding to positions 11,508-11,527 of the gene (Supplementary
359 Fig. 2c-d). The guide was cloned in the pDC2-Cas9-U6-hdhfr⁴⁵ plasmid to
360 obtain pDC2_ *pfap2hs*_sgRNA-C. The donor plasmid for tagging with eYFP
361 (pHR-C_ *pfap2hs*_eYFP) was based on plasmid pHRap2g-eYFP⁴⁶, with the
362 *pfap2-g* homology regions and *hsp90* 3' sequence replaced by *pfap2-hs*
363 homology regions. The 5' homology region (HR1) was generated with a PCR-
364 amplified fragment spanning from nucleotide 10,964 to the sequence of the
365 guide (recodonized), and a 47 bp fragment (generated by annealing two
366 complementary oligonucleotides) consisting of a recodonized version of the
367 remaining nucleotides to the end of the gene. The two fragments were cloned
368 simultaneously, using the In-Fusion system, into *SpeI*-*BglII* sites. The 3'
369 homology region (HR2) was a PCR fragment spanning position +1 to +590 after
370 the *pfap2-hs* STOP codon. It was cloned into *XhoI*-*AatII* restriction sites. The
371 donor plasmid for 3xHA C-terminal tagging (pHR-C_ *pfap2hs*_3xHA_ *hsp90*-3')
372 was also based on plasmid pHRap2g-eYFP⁴⁶, with the eYFP coding sequence

373 replaced by the 3xHA sequence (amplified from plasmid pHH1inv-pfap2-g-
374 HAx3¹⁴) and the same homology regions as in plasmid pHR-C_pfap2hs_eYFP
375 (but HR2 was cloned, using the In-Fusion system, into *EcoRI*-*AatII* sites,
376 because in this construct the *hsp90* 3' region in pHRap2g-eYFP was
377 maintained).

378
379 For N-terminal tagging (10E_*pfap2-hs*_eYFP-Nterm line), we cloned a guide
380 targeting *pfap2-hs* positions 73-92 in the pDC2-Cas9-U6-hDHFRyFCU⁴⁷
381 plasmid to obtain plasmid pDC2_pfap2hs_sgRNA-N (Supplementary Fig. 2e).
382 The donor plasmid (pfap2hs_HR-N_eYFP) consisted of a 5' homology region
383 (HR1) including positions -366 to -1 relative to the *pfap2-hs* start codon, the
384 eYFP gene and an in frame 3' homology region (HR2) spanning positions 4-756
385 of the gene (excluding the ATG) in which the nucleotides up to the position of
386 the guide were recodonized. HR1 and HR2 were cloned into *SacII*-*NcoI* and
387 *SpeI*-*EcoRI* sites, respectively. HR2 was amplified in two steps using a nested
388 PCR approach to add the recodonized sequences. The eYFP fragment (PCR-
389 amplified from plasmid pHR-C_pfap2hs_eYFP) was cloned using the In-Fusion
390 system into *SpeI*/*NcoI* sites. All oligonucleotides used to generate the plasmids
391 are described in Supplementary Table 5.

392
393 Transfections were performed by electroporation of ring stage cultures with 100
394 µg of plasmid (HA-ddFKBP tagging) or with a mixture of 12 µg linearized donor
395 plasmid and 60 µg of circular Cas9 plasmid (CRISPR-Cas9 system).
396 Linearization was achieved by digestion with the *PvuI* restriction enzyme
397 (cleaving the *amp* resistance gene of the donor plasmid). Transfected cultures

398 were selected with 10 nM WR99210 for four days as previously described⁴⁷
 399 (transfections using the CRISPR-Cas9 system), or with continuous WR99210
 400 pressure until parasites were observed, followed by 3 off/on drug cycles and
 401 subcloning by limiting dilution (transfections with the *pfap2hs*_HA-ddFKBP
 402 plasmid). In all cases, to assess correct integration we used analytical PCR of
 403 genomic DNA (Supplementary Fig. 2) with specific primers (Supplementary
 404 Table 5).

405
 406 **HS resistance assay.** HS was always performed on cultures at the mature
 407 trophozoite stage unless otherwise indicated. To measure survival to HS,
 408 cultures were tightly synchronized to a defined age window, diluted to 1%
 409 parasitaemia, split in two identical petri dishes (HS and control) maintained in
 410 independent air-tight incubation chambers, and exposed to HS when the
 411 majority of parasites were at the mature trophozoite stage (typically ~30-35 h
 412 post-invasion, hpi; Δ *pfap2-hs* lines were tightly synchronized 3 h earlier than the
 413 other lines but exposed to HS in parallel to account for their slower IDC
 414 progression). The exception was experiments to screen many subclones (i.e.,
 415 Fig. 1e) or to characterize transgenic parasite lines (i.e., Supplementary Fig. 2),
 416 in which cultures were only sorbitol-synchronized and HS performed ~20-25 h
 417 after sorbitol lysis (mature trophozoite stage). For HS, the full incubation
 418 chamber was transferred to an incubator at 41.5°C for 3 h, and then placed
 419 back to 37 or 35°C (the latter temperature was used for all lines in experiments
 420 including the *pfap2-hs* knockout lines). The chamber with the control cultures
 421 was always maintained at 37 or 35°C. After reinvasion (typically ~60-70 h after
 422 synchronization to ensure that all parasites had completed the cycle, including

423 parasites subjected to HS that show delayed progression through the IDC),
 424 parasitaemia of control and HS-exposed cultures was measured by flow
 425 cytometry using a FACScalibur flow cytometer (Becton Dickinson) and SYTO
 426 11 to stain nucleic acids, as previously described⁴⁸.

427

428 **Phenotypic characterization.** To determine the growth rate (increase in
 429 parasitaemia between consecutive cycles) at different temperatures, the
 430 parasitaemia of sorbitol-synchronized cultures was adjusted to 1% and then
 431 accurately determined by flow cytometry. Cultures were then split between two
 432 or three dishes maintained in parallel in incubators at the different temperatures
 433 tested. Parasitaemia was again determined by flow cytometry at the next cycle
 434 to determine the growth rate. To measure the duration of the IDC (at 35°C) in
 435 the different parasite lines we used a recently described method based on
 436 synchronization to a 1 h age window achieved by Percoll-purification of
 437 schizonts followed by sorbitol lysis 1 h later⁴⁸. The determination of the number
 438 of merozoites per fully mature schizont was based on light microscopy analysis
 439 of Giemsa-stained smears from Percoll-purified schizonts⁴⁸. DHA (Sigma no.
 440 D7439) or epoxomicin (Selleckchem no. S7038) sensitivity was measured by
 441 exposing tightly synchronized cultures at the ring (10-15 hpi, DHA only) or
 442 trophozoite (30-35 hpi, DHA or epoxomicin) stage (1% parasitaemia) to different
 443 concentrations of the compounds for 3 h. Parasitaemia was measured by light
 444 microscopy analysis of Giemsa-stained smears at the next cycle (typically 70-80
 445 h after Percoll-sorbitol synchronization). For these experiments, the *Δpfap2-hs*
 446 lines were tightly synchronized 3 h earlier than the other lines but exposed to

447 DHA or epoxomicin in parallel (13-18 or 33-38 hpi), to account for their slower
448 IDC progression.

449

450 **Transcriptional analysis by reverse transcription–quantitative PCR (RT-**
451 **qPCR).** RNA from tightly synchronized cultures exposed to HS and their
452 controls was obtained using the Trizol method, DNase-treated and purified
453 essentially as described⁴⁹. Reverse transcription and qPCR analysis of cDNAs
454 were also performed as described before⁴⁹. In brief, a mixture of random
455 primers and oligo (dT) were used for reverse transcription, and for qPCR we
456 used the PowerSYBR Green Master Mix (Applied biosystems) and the standard
457 curve method (each plate included a standard curve for each primer pair). All
458 primers used are listed in Supplementary Table 5. Unless otherwise indicated,
459 transcript levels were normalized against *serine--tRNA ligase*
460 (PF3D7_0717700), which shows relatively stable expression throughout the
461 IDC.

462

463 **Transcriptomic analysis using microarrays.** To compare the transcriptome of
464 control and HS-adapted 3D7-A parasite lines across the IDC we used
465 previously described two-colour long oligonucleotide-based glass microarrays¹⁹.
466 RNA was obtained from tightly synchronized cultures (5 h age window) at 8-13,
467 16-21, 24-29, 32-37 and 40-45 hpi. All samples (Cy5-labeled) were hybridized
468 together with a reference pool (Cy3-labeled) consisting of a mixture of equal
469 amounts of cDNA from rings, trophozoites and schizonts from control and HS-
470 adapted lines. Comparative genome hybridization was used to identify potential
471 transcript level differences attributable to genetic deletions or duplications.

472 5,142 genes produced useful data. Sample preparation, microarray
 473 hybridization and data analysis were performed essentially as described¹⁹.
 474
 475 To analyse the transcriptome of 10E, 10G and 10E_Δ*pfap2-hs* parasite lines
 476 under control and HS conditions, we used two-colour long oligonucleotide-
 477 based custom Agilent microarrays⁵⁰. The microarray design was based on
 478 Agilent design AMADID no. 037237^{50,51}, but we modified it as previously
 479 described (new design AMADID no. 084561)⁵². RNA was obtained from cultures
 480 synchronized to a 5 h age window at a ~2.5% parasitaemia. Given the slower
 481 IDC progression of 10E_Δ*pfap2-hs*, cultures of this parasite line were
 482 synchronized to 0-5 hpi 3 h earlier than 10E and 10G cultures, such that at the
 483 time of starting HS (in parallel for all lines) all cultures were approximately at the
 484 same stage of IDC progression. HS was started at 30-35 hpi (33-38 hpi for the
 485 10E_Δ*pfap2-hs* line) and samples collected before, during and after HS as
 486 indicated. RNA was prepared using the Trizol method. Sample preparation and
 487 microarray hybridization were performed essentially as described⁵¹. All samples
 488 (Cy5-labeled) were hybridized together with a reference pool (Cy3-labeled)
 489 consisting of a mixture of equal amounts of cDNA from rings, trophozoites and
 490 schizonts from 3D7-A. Microarray images were obtained using a DNA
 491 Microarray Scanner (no. G2505C, Agilent Technologies) located in a low ozone
 492 area, and initial data processing was performed using the GE2_1105_Oct12
 493 extraction protocol in Agilent Feature Extraction 11.5.1.1 software.
 494
 495 Agilent microarray data was analysed using Bioconductor in an R environment
 496 (R version 3.5.3). For each individual microarray, we calculated Cy3 and Cy5

background signal as the median of the 100 lowest signal probes for each channel, and probes with both Cy3 and Cy5 signals below three times the array background were excluded. Gene level $\log_2(\text{Cy5/Cy3})$ values, statistical estimation of parasite age⁵³ and estimation of average expression fold-differences across a time interval (for the comparison between parasite lines in the absence of HS) were performed as described¹⁹. The $\log_2$ of the expression fold-change upon HS was calculated, for each gene and time point, as the $\log_2(\text{Cy5/Cy3})$ in the HS-exposed sample minus the $\log_2(\text{Cy5/Cy3})$ in the control sample at the same parasite age, calculated using linear interpolation in the $\log_2(\text{Cy5/Cy3})$ versus estimated age plot. Visual inspection was used to exclude from further analysis genes with apparent artefacts. Genes missing data for ≥ 2 time points (or ≥ 1 for the comparison between parasite lines in the absence of HS across a time interval), or with values within the lowest 15th percentile of expression intensity (Cy5 sample channel) in all samples, were also excluded from further analysis. 4,964 genes produced useful data.

512

To assess the level of similarity to a reference non-stressed transcriptome (HB3 line) with high temporal resolution²³ we calculated the Pearson correlation between each sample and the time point with which it has higher similarity. Heatmaps and hierarchical clustering based on Spearman (Fig. 2) or Pearson (Supplementary Fig. 6) correlation were generated using Multiple Experiment Viewer (MeV) 4.9⁵⁴. Expression trend plots for each cluster were generated using ggplot2, with LOESS smoothing, and Venn diagrams using the eulerr package (both in an R environment). Motif analysis (5 to 8 bp) was performed using MEME 5.0.3 software. Functional enrichment analysis using GO terms

522 annotated in PlasmoDB version 43 was performed using Ontologizer 2.1
523 software⁵⁵ with the topology-elim method⁵⁶. Gene set enrichment analysis
524 (GSEA) was performed using GSEA v3.0 Preranked⁵⁷.

525

526 **Whole-genome sequencing analysis and analysis of publicly available**
527 **genome sequences from field isolates.** To sequence the full genome of
528 control and HS-adapted 3D7-A lines (two biological replicates), we used PCR-
529 free whole-genome Illumina sequencing. In brief, genomic DNA was sheared to
530 ~150-400 bp fragments using a Covaris S220 ultrasonicator and analysed using
531 an Agilent 2100 Bioanalyzer. For library preparation we used the NEBNext DNA
532 Library Prep Master Mix Set for Illumina (no. E6040S) using specific paired-end
533 TruSeq Illumina adaptors for each sample. After quality check by qPCR, we
534 obtained >6 million 150 bp paired reads for each sample using an Illumina
535 MiSeq sequencing system. After checking reads quality (FastQC algorithm) and
536 trimming adaptors (Cutadapt algorithm), sequence reads were mapped to the
537 PlasmodDB *P. falciparum* 3D7 reference genome version 24
538 (<https://plasmodb.org/plasmo/>) using the Bowtie2 local alignment algorithm.
539 Variant calling was performed using GATK-UnifiedGenotyper and following a
540 pipeline based on GATK best practices to identify SNPs and small indels.
541 Variants with low calling quality (Phred QUAL<20) and low read depth (DP<10)
542 were filtered out using GATK-VariantFiltration, and only variants present in both
543 biological replicates were considered. Differences in SNP/indel frequency
544 between control and HS-adapted lines were calculated for each SNP/indel, and
545 those showing <25% difference in any of the two replicates were filtered out.
546 Genome Browse (Golden Helix) was used to visualize alignments and variants.

547

548 For the analysis of publicly available genome sequences, we used the Pf3K
549 Project (2016): pilot data release 5 (www.malariagen.net/data/pf3k-5) containing
550 the sequence of >2,500 field isolates. Only SNPs that passed all quality filters
551 and did not fall within a region with multiple large insertions and deletions were
552 considered to be high-confidence. Using these criteria, a single high-confidence
553 polymorphism was identified at the *pfap2-hs* gene (producing the C3168X
554 mutation that results in a truncated PfAP2-HS protein that lacks D3).

555

556 **ChIP experiments and data analysis.** For ChIP experiments, synchronous 50
557 ml cultures at 2.5 to 5% parasitemia were harvested at the mid trophozoite
558 stage. For replicates in which ChIP was performed in parallel under HS and
559 control conditions, cultures were split off from a single parent flask at the mid
560 trophozoite stage. Control flasks were immediately returned to 37°C whereas
561 HS flasks were maintained at 41.5°C for 3 h before harvesting for ChIP
562 analysis.

563

564 ChIP followed by qPCR or Illumina sequencing was performed as described⁵⁸
565 using the 3F10 rat anti-HA antibody (1:500; Roche no. 11867423001) to
566 immunoprecipitate HA tagged AP2-HS, with the following modification: total
567 chromatin was diluted 5-fold in dilution buffer following sonication. The Illumina
568 HiSeq system was used to obtain 125 bp paired-end (replicates 1-3) or 150 bp
569 single-end (replicates 4-5) reads.

570

ChIP-seq data analysis was performed essentially as described⁵⁸. In brief, after trimming, quality control, mapping the remaining reads to the *Plasmodium falciparum* genome (PlasmoDB release 28) using BWA-MEM and filtering duplicated reads, peak calling was performed using MACS2⁵⁹ with a q-value cut-off of 0.01. Conversion to log₂ coverage of immunoprecipitate/input was performed using DeepTools BamCompare, selecting the paired end parameter for all tools when analyzing experiments including control and HS conditions. Overlapping intervals within called peaks for each dataset were determined using Bedtools MultIntersect. The closest annotated gene coding sequence for each called peak was determined using Bedtools ClosestBed. To visualize aligned data, we used IGV.

ChIP samples were analysed by qPCR in triplicate wells with primers described in Supplementary Table 5. All primer pairs were confirmed to have between 80 and 110% efficiency using sheared genomic DNA as a template control. The percent input was calculated using the formula $100 \cdot 2^{(Ct_{\text{adjusted input}} - Ct_{\text{IP}})}$.

Western blot. Synchronized cultures at the mature trophozoite stage were exposed to a regular 3 h HS or to a 1.5 h DHA pulse (positive control for a condition known to produce proteotoxic stress and induce the UPR)^{25,32}. Parasites were obtained using saponin lysis (0.15% w/v saponin) and pellets solubilized in 1x SDS-PAGE loading buffer with 4% β-mercaptoethanol and boiled at 95 °C for 5 min. Proteins were resolved by SDS-PAGE on 4-20% TGX Mini-PROTEAN gels (Bio-rad) and transferred to nitrocellulose membranes (Bio-rad). After blocking with 5% (w/v) bovine serum albumin (Biowest) in TBS-

596 T (0.1% Tween 20 in tris buffered saline), membranes were incubated at 4°C
597 overnight with the following primary antibodies: rabbit anti-ubiquitin (1:1000; Cell
598 Signaling Technology no. 3933), rabbit anti-phospho-eIF2α (1:1000; Cell
599 Signaling Technology no. 3398) and rabbit anti-histone H3 (1:1000; Cell
600 Signaling Technology no. 9715). After incubation with a goat anti-rabbit IgG-
601 peroxidase (1:5000; Millipore no. AP307P) secondary antibody, peroxidase was
602 detected using the Pierce ECL Western Blotting Substrate (Thermo Fisher
603 Scientific) in an ImageQuant LAS 4000 imaging system. To control for equal
604 loading, parts of the membranes corresponding to different molecular weight
605 ranges were separately hybridized with different antibodies. Signal
606 quantification was performed using ImageJ.

607

608 **Statistical analysis.** *P* values were calculated using a two-tailed *t*-test (equal
609 variance). Only significant *P* values are shown in the figures. No statistical
610 analysis was performed for experiments involving only two replicates. In all
611 cases, *n* indicates independent biological replicates (i.e., samples were
612 obtained from independent cultures). For cell cycle duration and DHA or
613 epoxomicin survival experiments, values were fitted to a sigmoidal dose-
614 response curve.

615

616 **Code availability.** The scripts used for the analysis of microarray and next
617 generation sequencing data will be made available upon reasonable request,
618 without any restrictions.

619

Data availability. The microarray data presented in Fig. 2 and Supplementary Fig. 1, 5, 6 and 9 has been deposited to the Gene Expression Omnibus (GEO) database with accession code GSE149394. Genome sequencing and ChIP-seq data presented in Fig. 1a, Fig. 2 and Supplementary Fig. 7 have been deposited to the Sequence Read Archive (SRA) database with accession codes PRJNA626524 and PRJNA670721, respectively. The authors declare that all other relevant data generated or analysed during this study are included in the Article or the Supplementary Information files. Materials and protocols are available from the corresponding author on reasonable request.

629

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815

816 **AUTHOR CONTRIBUTIONS**

817 E.T.-F. performed all experiments except for those presented in Supplementary
 818 Fig. 1, Western blot and ChIP-seq experiments. L.M.-T., E.T.-F., T.J.R. and
 819 A.C. performed the bioinformatics analysis. N.C.-V. performed Western blot
 820 experiments. T.J.R. performed and M.L. supervised ChIP-seq experiments. Z.B.
 821 provided microarray hybridizations for experiments presented in Supplementary
 822 Fig. 1. D.J.C. advised on clinical isolates and provided Line 1 from The Gambia.
 823 E.T.-F. and A.C. conceived the project, designed and interpreted the
 824 experiments, and wrote the manuscript (with input from all authors and major
 825 input from M.L. and D.J.C.).

826

827 **COMPETING INTERESTS STATEMENT**

828 The authors declare no competing interests.

829

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833

834 FIGURE LEGENDS

835 **Fig 1. A premature STOP codon in PfAP2-HS determines HS sensitivity. a,**

836 Proportion of Illumina reads with or without a nonsense mutation in *pfap2-hs* in

837 two independent HS-adapted cultures (3D7-A-HS r1 and r2) and their controls

838 (3D7-A r1 and r2). **b,** Sanger sequencing confirmation of the mutation. **c,**

839 Schematic of wild-type PfAP2-HS, PfAP2-HS_ΔD3 and ΔPfAP2-HS. The

840 position of the AP2 domains is indicated (D1-3). **d,** HS survival at the

841 trophozoite stage of 3D7-A subclones carrying or not the Q3417X mutation

842 (mean of $n=2$). **e,** HS survival of tightly synchronized wild-type, 10G and

843 Δ*pfap2-hs* cultures. Values are the mean and s.e.m. of $n=5$ (10E and 10G lines)

844 or $n=3$ (HB3 and D10 lines).

845

846 **Fig 2. Global transcriptional alterations upon HS. a,** Hierarchical clustering

847 of genes with altered transcript levels (≥ 4 fold-change at any of the time points

848 analysed) during (1.5 and 3 h) or after (2 h post) HS. Values are the $\log_2$ of the

849 expression fold-change in HS versus control cultures. 13 genes had values out

850 of the range displayed (actual range: -4.78 to +4.93). For each cluster, mean

851 values (with 95% confidence interval) for the genes in the cluster and

852 representative enriched GO terms are shown. Columns at the left indicate

853 annotation as chaperone⁴, presence of the G-box¹⁰ or tandem G-box (TdGbox)

854 in the upstream region, and $\log_2$ fold-change during HS in a previous study²⁴

855 (Oakley). **b,** Venn diagrams of the genes altered upon HS in the three parasite

856 lines. **c**, Pearson correlation of the genome-wide transcript levels of each
857 culture versus the most similar time point of a high-density time-course
858 reference transcriptome²³. **d**, Age progression during the assay, statistically
859 estimated⁵³ from the transcriptomic data. **e**, ChIP-seq analysis (representative
860 of $n=5$ for 35°C and of $n=3$ for HS) of HA-tagged PfAP2-HS. The log₂-
861 transformed ChIP/input ratio at the *hsp70-1* and *hsp90* loci is shown. The
862 position of the tandem G-box is indicated.

863

864 **Fig 3. Phenotypic characterization of parasite lines lacking PfAP2-HS at**
865 **non-febrile temperatures. a**, Growth rate of $\Delta pfap2-hs$ and parental lines at
866 different temperatures (mean and s.e.m. of $n=4$). **b**, Number of merozoites per
867 schizont (median and quartiles box with 10-90 percentile whiskers). Values
868 were obtained from 100 schizonts for each parasite line and condition. **c**,
869 Duration of the asexual blood cycle. The cumulative percent of new rings
870 formed at each time point is shown (mean and s.e.m. of $n=2$). **d**, Survival (%)
871 after a 3 h dihydroartemisinin (DHA) pulse at the ring or trophozoite stage
872 (mean and s.e.m. of $n=3$). **e**, Survival (%) after a 3 h epoxomicin pulse at the
873 trophozoite stage. Values are the mean and s.e.m. of $n=4$ (100 nM) or $n=3$ (150
874 nM). **f**, Schematic model of the *P. falciparum* HS response, involving rapid
875 upregulation of the expression of a very restricted set of key chaperones by
876 PfAP2-HS. The PF3D7_1421800 gene (in brackets) shows PfAP2-HS-
877 dependent increased transcript levels upon HS, but PfAP2-HS binding was not
878 detected in its promoter, and it lacks a G-box. The main defects associated with
879 PfAP2-HS deletion or truncation, under HS or basal conditions, are listed.

880

Fig. 1.

Fig. 2.

Fig. 3.