SUPPLEMENTAL METHODS

Sample collection

P. falciparum-infected blood samples were obtained from returning travelers (≥18 years old) attending the clinics at the Institute of Tropical Medicine or University Hospital in Antwerp (Belgium), and from children (≤12 years old) attending the Brikama Health Centre (The Gambia) as part of an ongoing drug efficacy study. 1-3.5 ml of venous-blood were collected in citrate phosphate dextrose adenine (CPDA) or EDTA vacutainers. Parasites were placed in culture within 6 h of collection. In addition, cryopreserved parasite isolates obtained from children participating in previous studies at MRC Unit The Gambia (MRC biobank) were included in the study. We also analyzed blood samples collected from volunteers participating in a controlled human malaria infection (CHMI) study [1]. In brief, *P. falciparum* sporozoites of the NF54 parasite line at Sanaria were injected into malaria-naïve volunteers and venous blood samples were collected and cryopreserved at day 9 after sporozoites injection and at the day of malaria (when parasites are first detected by microscopy, 12-15 days post-injection).

Parasite cultures

Parasites were cultured at 3% hematocrit under standard conditions, with Albumax II or inactivated human serum according to regular procedures at each of the laboratories involved in this study. To verify that supplementing with Albumax II or inactivated serum does not affect *clag3* expression, transcript levels of these genes were compared between 3D7 cultures maintained in parallel under both conditions, which confirmed similar *clag3* expression patterns (Supplemental Fig. S1A). To prepare RNA for *clag* transcriptional analysis, cultures were harvested when the majority of parasites were at the schizont stage.

In the case of natural infections, parasites were cultured only until they reached the schizont stage of the first cycle, with the exception of two of the samples (FR39 and CR100), which had to be cultured for one additional cycle due to very low parasitaemia. Day 9 and day of malaria samples from the CHMI were cryopreserved and, after thawing, parasites were cultured for 2-3 weeks or ~1 week, respectively, until they reached a ≥0.02% parasitaemia. Cultures were then sorbitol-synchronized and harvested at the schizont stage. Selection with BS (ThermoFisher) was performed with a sub-lethal concentration of the drug (0.3 µg/ml) as previously described [2].

Genetic analysis

Genomic DNA (gDNA) was extracted using the QIAamp DNA Blood Mini Kit (Qiagen) following manufacturer's instructions. The multiplicity of infection of field samples was estimated as the highest number of alleles observed by genotyping the *msp1* and *msp2* loci. Amplification of the *msp1* and *msp2* target sequences was performed using fluorescently-labeled primers and analyzed by capillary electrophoresis approximately as previously described [3]. To assess for recombination events between the two *clag3* genes, the *clag3* loci were analyzed by long PCR with primers against the conserved paralog-specific regions flanking the genes, as previously described [4].

We also used *msp1* and *msp2* genotyping to confirm that samples P04 and P12 contained the same haplotype at different times along the selection experiments reported in Fig. 2 in the main manuscript, thus excluding the possibility that changes in *clag3* expression were associated with selection of genetically different parasites present in the population. We also excluded recombination between the two *clag3* genes [4] during adaptation.

For each isolate, we sequenced the hypervariable region (HVR) of each *clag3* gene from the long PCR products, using primers against the conserved regions around the HVR (Supplemental Table S1). The sequences obtained were used to design new gene- and isolate-specific primers or to determine which existing primers are suitable for the analysis of *clag3.1* and *clag3.2* transcript levels in each isolate. The full *clag3.1* and *clag3.2* genes from isolates P04 and P12 were sequenced from long PCR products using conventional Sanger sequencing.

Preparation of RNA, reverse transcription and quantitative real time PCR

RNA was purified approximately as described [5]. In brief, parasites were collected in Trizol (Invitrogen) and, after phase separation, RNA was purified using the RNeasy Mini Kit (Qiagen), following the supplier instructions except for DNase digestion that was performed on-column. RNA was reverse transcribed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). To exclude the possibility of gDNA contamination, parallel reactions were performed in the absence of reverse transcriptase (-RT controls). The optimized protocol was validated for use at very low parasitaemias by comparing *clag3* expression in two parasite lines between 2% and 0.02% parasitaemia (Supplemental Fig. S1B). Quantitative PCR analysis of cDNAs and -RT controls was performed in triplicate wells using the Power SYBR Green Master Mix (Applied Biosystems) or SensiMix SYBR No-ROX Kit (Bioline). Each 96-well plate contained a standard curve made with serial dilutions of 3D7 gDNA, except in the case of *clag3* analysis of field isolates for which the standard curve was prepared with gDNA from the same isolate. Expression values, in arbitrary units, were calculated using the standard curve method as previously described [6]. Expression values of *clag* genes were normalized against expression of *rhoph2* (PF3D7_0929400), which

shows a similar temporal expression pattern. We also measured expression of the constitutive gene *seryl tRNA synthetase* (*seryl*, PF3D7_0717700) to estimate the proportion of parasites at the schizont stage. Samples with a *rhoph2/seryl* ratio <2 were excluded from further analysis based on our previous observations that well-synchronized cultures with a predominance of schizonts show higher values of this ratio. In spite of using *rhoph2* for normalization and always harvesting cultures for transcriptional analysis at a similar stage of parasite development, total normalized *clag3* transcript levels (*clag3.1* + *clag3.2* transcript levels) show intrinsic variability that may not reflect actual biological differences, especially among samples from patients or volunteers that had to be analyzed at very low parasitaemia after minimal in vitro culture and could not be tightly synchronized. Variability in total normalized *clag3* transcript levels, which sometimes is observed even between biological replicates, is probably attributable to small differences in the temporal expression pattern between *rhoph2* and *clag3* genes and to small differences in life cycle progression between samples. Hence, we drew our conclusions from the analysis of the relative abundance of *clag3.1* and *clag3.2* transcripts, which can be measured accurately and is highly reproducible.

For *clag3* genes we used paralog- and isolate-specific primers, whereas for other *clag* genes we designed primers against conserved regions in which no polymorphism is reported at www.malariagen.net [7] (Supplemental Table S1). Absence of polymorphism was also confirmed by sequencing these regions in some of the isolates.

CLAG3 sequences analysis

Publicly available CLAG3.1 and CLAG3.2 full length or HVR sequences were obtained from PlasmoDB (www.plasmodb.org) and GenBank. Alignments were performed with ClustalW and manual refinement. Pairwise sequence distances were computed using the Jones-Taylor-

Thorton substitution model with a gamma distribution (shape parameter = 1) as implemented in MEGA6 [8]. A phylogenetic tree was then generated in MEGA6 using the Neighbor-Joining method with pairwise removal of gaps and 500 bootstrap replicates to assess topology reliability. An unrooted radial cladogram was generated using FigTree v1.4.2 (http://tree.bio.ed.ac.uk), whereas split networks of CLAG3 sequences were inferred using SplitsTree4 (www.splitstree.org) [9].

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