Supplementary Information for

PIMMS43 is required for malaria parasite immune evasion and sporogonic development in the mosquito vector

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Figure S1. Multiple sequence alignment of Plasmodium PIMMS43 orthologs

Sequence alignment of PIMMS43 orthologs in six Plasmodium species: P. falciparum (PfPIMMS43; PF3D7_0620000), P. berghei (PbPIMMS43; PBANKA_1119200), P. yoelii (PyPIMMS43; PYYM_1121200), P. chabaudi (PcPIMMS43; PCHAS_1118700), P. knowlesi (PknPIMMS43; PKNH_1130300) and P. vivax (PvPIMMS43; PVX_114125). Amino acid residues are color-shaded according to their biochemical characteristics. Conserved Cysteine residues are indicated with arrows. The two variable regions are highlighted with grey background color. In the consensus sequence, dots and colons mark conserved amino acid residues with weakly and strongly similar properties, respectively, and asterisks mark amino acid residues that are identical between orthologs. Protein sequences were retrieved from PlasmoDB.
Figure S2. PIMMS43 RT-PCR transcription profiles

(A) RT-PCR analysis of Pfc43 transcripts in in vitro and in vivo NF54 parasite populations. Gametocyte expressed gene Pfs25 and sporozoite-expressed gene PfCSP serve as both stage-specific and loading controls. (B) RT-PCR analysis of the expression of Pbc43 transcripts in blood stages, in vitro ookinetes and A. coluzzii mosquito stages. Gametocyte expressed gene P28 and constitutive expressed GFP served as a stage-specific and loading controls, respectively. Δc43 parasites were used as a negative control. Abbreviations: ABS, asexual blood stages; NGP, non-gametocyte producing; MBS, mixed blood stages; Gc, gametocytes; Gc (+), activated gametocytes; Ook, ookinetes; pbf, post blood feeding; wt, wild-type (c507); ko, Δc43 knockout.
Figure S3. Generation of *P. berghei Δc43* knock out mutant in the *c507* reference line

(A) Schematic representation of *Pbc43* locus disruption by double crossover homologous recombination. The disruption vector design allows for 50% ko of the coding DNA sequence. Arrows indicate binding sites for primers P5, P6 and P7 used in diagnostic PCR. P5 and P7 were used to detect integration and P5 and P6 that bind to the endogenous *PbPIMMS43* locus were used to confirm absence of the endogenous gene in the ko line. (B-C) Genotypic analysis of Δc43 mutants following transfection and dilution cloning by (B) southern blot analysis on pulsed field gel electrophoresis separated transgenic chromosomes and (C) PCR on mix parasite (MP) and Δc43 clonal populations (CP). Used primer combinations are shown below each detected band.
Figure S4. Generation and phenotypic analysis of PbPfc43

(A) Schematic diagram of the endogenous Pbc43 locus, targeting construct and resulting transgenic PbPfc43 locus. Arrows P6, P20 and P21 indicate binding sites for primers used in diagnostic PCR. P20 and P21 were used to detect integration, and P20 and P6 that bind to the endogenous Pbc43 locus were used to confirm absence of the endogenous gene in the transgenic line. (B) Diagnostic PCR to determine integration of the Pfc43 targeting construct into the endogenous Pbc43 locus. The c507 wt parasite served as a control. (C) RT-PCR in ookinetes to confirm expression of Pfc43 in the PbPfc43 transgenic line. Amplifications of Pbc43 and CTRP were used as positive control for the endogenous locus and stage-specific control, respectively. (D) Western blot analysis under reducing and no-reducing conditions of whole c507, Δc43 and PbPfc43 parasite cell lysates using the α-Pbc43opt and α-Pfc43opt antibodies. Pfc43 protein bands are indicated with asterisks. (E) Oocyst density in naïve and LRIM1 kd A. coluzzii infected with the PbPfc43 transgenic line. The c507 wt and Δc43 parasites served as controls in naïve mosquito infections. Red horizontal lines indicate median. ***, P<0.0001 with Mann-Whitney test.
Figure S5. PIMMS43 localization on the surface of young oocysts and midgut sporozoites in *A. coluzzii* infected midgut epithelia

(A) Immunofluorescence assays of NF54 *P. falciparum* oocysts found in the mosquito midgut epithelium at 2 dpbf (left) and midgut/oocyst sporozoites (spz) at 11 dpbf (right), stained with α-Pfc43opt (green), α-Pfs25 (red) and/or α-PfCSP (purple) antibodies. DNA was stained with DAPI. Staining with pre-immune serum was used as a negative control. (B) Immunofluorescence assays of *P. berghei c507* parasites found in the mosquito midgut epithelium at 2 dpbf (left) and oocyst sporozoites at 15 dpbf (right), stained with α-Pbc43opt antibody (green), α-P28 (red) and/or α-PbCSP (purple) antibodies. DNA was stained with DAPI. Staining with pre-immune serum was used as a negative control. Images in both panels are de-convoluted projection of confocal stacks. BF denotes bright field and scale bars correspond to 5 μm.
Figure S6. Asexual blood stage (ABS) growth of Δc43 and control c507 parasites
ABS growth is measured every 24 hours upon parasite inoculation for 3 consecutive days and expressed as percentage (%) of infected red blood cells (iRBC). The arithmetic mean of two biological replicates and the standard error of the mean (SEM) are shown.
Figure S7. Complementation of Δc43 mutant with endogenous PbPIMMS43 wt allele

(A) Schematic diagram of the Δc43 ko locus, targeting construct and transgenic Δc43::c43wt complemented locus. Arrows indicate binding sites of primers used in diagnostic PCR. P20, P6 and P26 detect integration and are used to confirm re-introduction of the wt PbC43 in the Δc43::c43wt transgenic line. P20 and P7 bind to the Δc43 ko locus. (B) Diagnostic PCR to determine integration of the targeting construct into the Δc43 ko locus. The c507 wt and Δc43 parasites served as PCR controls. (C) Oocyst density in A. coluzzii infected with the Δc43::c43wt transgenic line. The c507 wt and Δc43 parasites served as controls. Red horizontal lines indicate the median; ns, not significant; ***, P<0.0001 with Mann-Whitney test.
Figure S8. Generation and phenotypic analysis of \textit{P. berghei} Δ\textit{c43}\textsuperscript{red} mutant line

\textbf{(A)} Schematic representation of \textit{PbPIMMS43} (\textit{Pbc43}) disruption using the PlasmoGEM vector, PbGEM: 042760. The design allows for 74\% removal of the gene coding sequence. Arrows indicate binding sites of primers P8-11 used in diagnostic PCR assays. P10 and P11 were used to detect integration, and P8 and P9 bind to the endogenous locus and were used to confirm absence of the endogenous gene in the ko parasite. \textbf{(B)} Diagnostic PCR to determine integration of the targeting construct into the endogenous locus. \textbf{(C)} Female gametocyte to ookinete conversion rate \textit{in vitro} (left), and \textit{in vivo} in the \textit{A. coluzzii} midgut (middle left) of \textit{P. berghei} c1804 and mutant Δ\textit{c43}\textsuperscript{red} lines. Error bars indicate SEM. Representative images of \textit{in vivo} invading and \textit{in vitro} produced Δ\textit{c43}\textsuperscript{red} ookinetes are shown (middle right). The graph on the right shows numbers of c1804 and mutant Δ\textit{c43}\textsuperscript{red} oocysts in the midguts of \textit{A. coluzzii} mosquitoes 10 dpbf. \textbf{(D)} Gametocyte to ookinete conversion rate in the midgut bolus (left) and oocyst numbers (right) of c1804 and Δ\textit{c43}\textsuperscript{red} \textit{P. berghei} lines in \textit{A. stephensi} mosquitoes. \textbf{(E)} Sporozoite numbers of c1804 and Δ\textit{c43}\textsuperscript{red} \textit{P. berghei} lines in the midgut of \textit{A. stephensi} mosquitoes. In all the graphs, red lines indicate median, \(n\) is the number of independent experiments, ns and *** denote non-significant P values and P<0.0001, respectively, assessed with the Mann-Whitney test.
Figure S9. *P. berghei* and *A. coluzzii* gene expression 1 hpf

(A) Volcano plot of *P. berghei* gene expression in Δc43 vs. c507 wt parasite lines in the *A. coluzzii* midgut. (B) Volcano plot of *A. coluzzii* midgut transcriptional responses to Δc43 vs. c507 wt parasites. X-axes show log2 fold change and y-axes show log10 p-value calculated using one-way ANOVA. Blue and orange filled circles indicate genes that are at least 2-fold down downregulated and 2-fold upregulated, respectively. Black circles show with no significant differential regulation. Known gene names are indicated.
Figure S10. Transcriptional profiles of TEP1, LRIM1 and APL1C in infected A. coluzzii tissues

Relative abundance of TEP1, LRIM1 and APL1C transcripts in the midgut and whole body of A. coluzzii mosquitoes infected with the c507, Δc43 or Δpbp47 parasite lines, measured by qRT-PCR at 24 hpbf. Whole body refers to mosquito tissues after the removal of wings, legs and head. Data are derived from two independent replicates, normalized to the abundance of mosquito S7 transcripts in each of the two tissues and referenced to data obtained at 1 hpbf that was used as baseline. Error bars indicate SEM.
Figure S11. Population genetic analysis of *Pfs47* in African *P. falciparum*

*Pfs47* fixation index (*F*<sub>ST</sub>) values of 1,509 *P. falciparum* populations sampled from patients across Africa (top panel) and schematic representation of SNPs with high *F*<sub>ST</sub> values leading to amino acid substitutions in each deduced protein (bottom panel). In top panel, colour coding indicates comparisons between countries in West, Central and East Africa. Central Africa includes populations sampled only from the Democratic Republic of the Congo. White bars overlaid with coloured bars indicate the *F*<sub>ST</sub> of *PfPIMMS43*. In bottom panel, boldfaced amino acid substitutions are those deriving from SNPs with total *F*<sub>ST</sub>&gt;0.1, and the rest of the substitutions are those showing high *F*<sub>ST</sub> in comparisons between populations sampled from specific countries. Substitutions presented in grey do not show high *F*<sub>ST</sub> but have been shown previously to be present in laboratory NF54 *P. falciparum* and be involved in parasite immune evasion. Substitutions marked with red stars are those showing very high *F*<sub>ST</sub> and have swept to almost fixation in some populations. Yellow spikes show the positions of conserved Cysteine residues. Burkina Faso, BF; Democratic Republic of the Congo, DC; Gambia, GM; Ghana, GH; Guinea, GN; Kenya, KE; Madagascar, MG; Malawi, MW; Mali, ML; Tanzania, TZ; Uganda, UG.
Table S1. Oocyst numbers in *A. coluzzii* infections

| Dataset          | Days post infection | Parasite | Number of experiments | Number of midguts | Prevalence (%) | Arithmetic mean | Median | Parasite range | P value |
|------------------|---------------------|----------|-----------------------|-------------------|----------------|----------------|--------|----------------|---------|
| I (Figure 3C)    | 3dpi                | *c507* Pool | 60                    | 88                | 134.6          | 112            | 0-453  |                | <0.0001 |
|                  |                     | Δc43      | 63                    | 0                 | 0              | 0              | 0      |                |         |
|                  |                     | *c507* R1 | 27                    | 93                | 132.4          | 99             | 0-378  |                | <0.0001 |
|                  |                     | Δc43      | 33                    | 0                 | 0              | 0              | 0      |                |         |
|                  |                     | *c507* R2 | 33                    | 85                | 136.5          | 123            | 0-453  |                | <0.0001 |
|                  |                     | Δc43      | 30                    | 0                 | 0              | 0              | 0      |                |         |
|                  | 5dpi                | *c507* Pool | 62                    | 90                | 97.7           | 61             | 0-435  |                | <0.0001 |
|                  |                     | Δc43      | 58                    | 0                 | 0              | 0              | 0      |                | <0.0001 |
|                  |                     | Δc43      | 26                    | 0                 | 0              | 0              | 0      |                | <0.0001 |
|                  |                     | *c507* R2 | 29                    | 93                | 85.2           | 59             | 0-362  |                | <0.0001 |
|                  |                     | Δc43      | 32                    | 0                 | 0              | 0              | 0      |                | <0.0001 |
|                  |                     | *c507* R2 | 62                    | 90                | 97.7           | 61             | 0-435  |                | <0.0001 |
|                  | 7dpi                | *c507* Pool | 63                    | 78                | 44.6           | 45             | 0-136  |                | <0.0001 |
|                  |                     | Δc43      | 56                    | 0                 | 0              | 0              | 0      |                | <0.0001 |
|                  |                     | Δc43      | 32                    | 75                | 41.9           | 34             | 0-136  |                | <0.0001 |
|                  |                     | Δc43      | 26                    | 0                 | 0              | 0              | 0      |                | <0.0001 |
|                  |                     | *c507* R2 | 31                    | 81                | 47.4           | 47             | 0-104  |                | <0.0001 |
|                  |                     | Δc43      | 30                    | 0                 | 0              | 0              | 0      |                | <0.0001 |
|                  | 10dpi               | *c507* Pool | 86                    | 98                | 52.6           | 47             | 0-164  |                | <0.0001 |
|                  |                     | Δc43      | 86                    | 0                 | 0              | 0              | 0      |                | <0.0001 |
|                  |                     | *c507* R1 | 30                    | 100               | 38.6           | 26             | 8-164  |                | <0.0001 |
|                  |                     | Δc43      | 30                    | 0                 | 0              | 0              | 0      |                | <0.0001 |
|                  |                     | *c507* R2 | 24                    | 92                | 63.4           | 63             | 0-142  |                | <0.0001 |
|                  |                     | Δc43      | 24                    | 0                 | 0              | 0              | 0      |                | <0.0001 |
|                  |                     | *c507* R3 | 32                    | 100               | 57.6           | 57             | 1-149  |                | <0.0001 |
|                  |                     | Δc43      | 32                    | 0                 | 0              | 0              | 0      |                | <0.0001 |
| II (Figure S5D)  | 10dpi               | *c507* Pool | 84                    | 69                | 19             | 6              | 0-184  |                | <0.0001 |
|                  |                     | Δc43      | 49                    | 59                | 18             | 6              | 0-91   | 0.8826         |         |
|                  |                     | Δc43      | 51                    | 0                 | 0              | 0              | 0      |                | <0.0001 |
|                  |                     | *c507* R1 | 42                    | 62                | 18.6           | 6              | 0-133  |                | <0.0001 |
|                  |                     | Δc43      | 25                    | 60                | 17.8           | 10             | 0-86   | 0.7279         |         |
|                  |                     | Δc43      | 27                    | 0                 | 0              | 0              | 0      |                | <0.0001 |
|                  |                     | Δc43      | 42                    | 76                | 18.8           | 5              | 0-184  |                | <0.0001 |
|                  |                     | Δc43      | 24                    | 58                | 18.5           | 4              | 0-91   | 0.5823         |         |

Oocyst data in the midgut of *A. coluzzii* mosquitoes infected with the *c507*, Δc43 or Δc43::c43wt *P. berghei* lines. Data from pooled and independent biological replicates are presented. *P* values were calculated using Mann-Whitney test. Datasets I and II are independent of each other.
| Parasite | Replicates | Midgut sporozoites | Salivary gland sporozoites | Infectivity to mice |
|----------|------------|--------------------|---------------------------|-------------------|
|          |            | Mean               | SEM                       | Mean              | SEM      | Day 21  |
| c507     | Pool       | 2,249              | 473                       | 2,146             | 235      | 9 of 9  |
| Δc43     |            | 0                  | 0                         | 0                 | 0        | 0 of 9  |
| c507     | R1         | 1,990 (2,100/1,850/2,020) | 60 | 2,715 (2,982/2,447) | 189 | 3 of 3 |
| Δc43     |            | 0                  | 0                         | 0                 | 0        | 0 of 3  |
| c507     | R2         | 1,400 (1,650/1,118/1,430) | 126 | 1,788 (2,361/1,215) | 405 | 3 of 3 |
| Δc43     |            | 0                  | 0                         | 0                 | 0        | 0 of 3  |
| c507     | R3         | 3,358 (2,504/4,070/3,500) | 374 | 1,936 (1,830/2,041) | 75 | 3 of 3 |
| Δc43     |            | 0                  | 0                         | 0                 | 0        | 0 of 3  |

Mean oocyst and salivary gland sporozoite numbers from three biological replicates of *A. coluzzii* infections with Δc43 or c507 parasite lines. For each biological replicate, sporozoite numbers was determined from two or three pools (technical replicates) of ten homogenized mosquito midguts or salivary glands at 15 and 21 days post infection respectively. Data from pooled and independent biological replicates are presented. The data from each technical replicate are also presented in brackets. Infectivity of sporozoites was assessed by infected mosquito bite back experiments with at least 30 mosquitoes on C57/BL6 mice at 21 dpi. Following this, parasitaemia was monitored until 14 days post mosquito bite. SEM represents standard error of mean.
| Parasite | Number of experiments | Number of midguts | Prevalence (%) | Arithmetic mean | Median | Parasite range | P value |
|----------|-----------------------|-------------------|----------------|----------------|--------|----------------|---------|
| c507     | Pool                  | 83                | 65             | 62.1           | 13     | 0-418          |         |
| Δc43     |                       | 79                | 62             | 30.3           | 7      | 0-403          | 0.0947  |
| wt       | R1                    | 35                | 46             | 27.0           | 2      | 0-107          |         |
| Δpbc43   |                       | 35                | 54             | 22.9           | 4      | 0-194          | 0.929   |
| wt       | R2                    | 32                | 75             | 71.4           | 45     | 0-281          |         |
| Δpbc43   |                       | 26                | 73             | 18.5           | 8      | 0-136          | 0.0543  |
| wt       | R3                    | 16                | 69             | 120.4          | 80     | 0-418          |         |
| Δpbc43   |                       | 18                | 61             | 61.6           | 14     | 0-403          | 0.1917  |

Numbers of melanised parasites detected in the midguts of c507 or Δc43 infected A. coluzzii mosquitoes at 4 days post infection. The p value was calculated using the Mann-Whitney U test. Data from pooled and independent biological replicates are presented.
Table S4. Effect of *A. coluzzii* gene silencing on oocyst numbers

| Dataset | Parasite | Mosquito | Number of experiments | Number of midguts | Prevalence (%) | Arithmetic mean | Median | Parasite range | P value |
|---------|----------|----------|-----------------------|-------------------|----------------|----------------|--------|----------------|---------|
| I (Figure 3F) | Δc43 | LacZ | Pool | 132 | 2 | 0.03 | 0 | 0-2 | <0.0001 |
| | LRIM1 | R1 | 40 | 8 | 0.1 | 0 | 0-2 | | |
| | LRIM1 | R2 | 32 | 0 | 0 | 0 | 0 | | |
| | LRIM1 | R3 | 39 | 74 | 18.50 | 10 | 0-119 | | <0.0001 |
| | LacZ | R4 | 40 | 0 | 0 | 0 | 0 | | |
| Δc43 | LacZ | Pool | 104 | 3 | 0.03 | 0 | 0-2 | <0.0001 |
| | TEP1 | R1 | 40 | 8 | 0.1 | 0 | 0-2 | | |
| | TEP1 | R2 | 37 | 86 | 28.03 | 8 | 0-525 | | <0.0001 |
| | LacZ | R3 | 35 | 66 | 27.01 | 6 | 0-146 | | <0.0001 |
| | TEP1 | | 32 | 0 | 0 | 0 | 0 | | |
| c507 | LacZ | Pool | 49 | 88 | 16.9 | 11 | 0-88 | | <0.0001 |
| | LRIM1 | R1 | 45 | 89 | 176.8 | 140 | 0-676 | | <0.0001 |
| | TEP1 | R2 | 23 | 96 | 71.1 | 56 | 0-286 | | <0.0001 |
| | LacZ | R3 | 21 | 90 | 16.76 | 11 | 0-56 | | |
| | TEP1 | | 24 | 96 | 192 | 190 | 0-676 | | <0.0001 |
| | LRIM1 | | 8 | 100 | 54.38 | 58 | 16-96 | | <0.005 |
| | LacZ | R2 | 28 | 86 | 17.07 | 11 | 0-88 | | |
| | LRIM1 | | 21 | 81 | 159.48 | 98 | 0-491 | | <0.005 |
| II (Figure S3E) | c507 | Naive | Pool | 54 | 73 | 10.3 | 4 | 0-47 | <0.0001 |
| | Pb/PfΔc43 | Naive | | 79 | 20 | 0.8 | 0 | 0-16 | | |
| | Δc43 | Naive | | 63 | 0 | 0 | 0 | 0 | | |
| | c507 | Naive | R1 | 42 | 64 | 8.8 | 2.5 | 0-47 | | <0.0001 |
| | Pb/PfΔc43 | Naive | | 67 | 16 | 0.6 | 0 | 0-16 | | |
| | Δc43 | Naive | | 43 | 0 | 0 | 0 | 0 | | |
| | c507 | Naive | R2 | 12 | 92 | 15.5 | 15.5 | 0-32 | | <0.0001 |
| | Pb/PfΔc43 | Naive | | 11 | 46 | 2.1 | 0 | 0-8 | | |
| | Δc43 | Naive | | 20 | 0 | 0 | 0 | 0 | | |
| III | Pb/PfΔc43 | LacZ | Pool | 77 | 13 | 0 | 0 | 0-5 | | |
(Figure S3E)

|        | Pb<sub>Δc43</sub> | LRIM1 | LacZ | R1  | 71   | 55  | 9   | 1   | 0-70 | <0.0001 |
|--------|-------------------|-------|------|-----|------|-----|-----|-----|------|---------|
| Pb<sub>Δc43</sub> | LRIM1             | 23    | 70   | 0.125 | 3   | 0-70 | <0.0001 |
| Pb<sub>Δc43</sub> | LacZ              | 25    | 8    | 0.12  | 0   | 0-2  |
| Pb<sub>Δc43</sub> | LRIM1             | 18    | 50   | 8.5  | 1   | 0-45 | 0.0003 |
| Pb<sub>Δc43</sub> | LacZ              | 28    | 18   | 0.42 | 0   | 0-5  |
| Pb<sub>Δc43</sub> | LRIM1             | 30    | 47   | 4.1  | 0   | 0-36 | 0.008  |

Numbers of Δc43, c507 or Pb<sub>Δc43</sub> oocysts in the midguts of naïve, LacZ or LRIM1 or TEP1 dsRNA injected A. coluzzii mosquitoes at 10 days post blood feeding on infected mice. P values were calculated using the Mann-Whitney U test. Datasets I, II and III are independent of each other. Data from pooled and independent replicates are presented.
| Parasite | Knockdown | Oocyst sporozoites | Salivary gland sporozoites | Infectivity to mice |
|----------|-----------|--------------------|---------------------------|--------------------|
|          |           | Mean              | SEM                       |                    |
| I. c507 vs. Δc43 sporozoite numbers in LacZ and LRIM1 kd mosquitoes |           |                   |                           |                    |
| c507     | LacZ      | 7,790             | 785                       | 8,622              | 673                | 6 of 6             |
| c507     | LRIM1     | 17,343            | 1,988                     | 16,060             | 665                | 6 of 6             |
| Δc43     | LacZ      | 0                 | 0                         | 0                  | 0                  | 0 of 10            |
| Δc43     | LRIM1     | 580               | 64                        | 65                 | 13                 | 0 of 11            |
| II. c507 vs. PbPfc43 sporozoite numbers in LacZ and LRIM1 kd mosquitoes |           |                   |                           |                    |
| c507     | LacZ      | 13,836            | 198                       | ND                 | ND                 | 2 of 2             |
| c507     | LRIM1     | 23,504            | 491                       | ND                 | ND                 | 1 of 1             |
| PbPfc43  | LacZ      | 346.86            | 9                         | ND                 | ND                 | 0 of 1             |
| PbPfc43  | LRIM1     | 456.41            | 13                        | ND                 | ND                 | 0 of 1             |

I. Mean oocyst and salivary gland sporozoite numbers in LacZ and LRIM1 knockdown A. coluzzii mosquitoes infected with c507 or Δc43 parasites, obtained from 2 to 4 biological replicates. In each replicate, the mean number of sporozoites was calculated from three pools of ten homogenised midguts or salivary glands at 15 and 21 days post blood feeding respectively. Parasite infectivity to mice was assessed upon blood feeding of at least 30 mosquitoes on C57BL/6 mice at 21-22 days post blood feeding. Parasitaemia was monitored until 14 days post mosquito bite. SEM represents the standard error of the mean.

II. The mean number of midgut sporozoites in LacZ and LRIM1 knockdown A. coluzzii mosquitoes infected with PbPfc43 or c507 parasites was calculated from two replicates obtained from suspensions of 10-12 homogenised midguts at day 15 post blood feeding. Parasitaemia was monitored until 14 days post mosquito bite.
Table S6. Sporozoite development and infectivity upon ookinete injection in the haemocoel

| Parasite | Mean Salivary gland sporozoites | SEM | Infectivity to mice |
|----------|--------------------------------|-----|---------------------|
| c507     | 6530 (2,934/10,117/8540)       | 1693| 6/6                 |
| Δc43     | 0 (0/0/0)                      | 0   | 0/6                 |

Mean salivary gland sporozoites at 21 days post *A. coluzzii* haemocoel inoculation with *c507* wt or Δc43 ookinetes, obtained from 3 biological replicates. Data from independent biological replicates are presented in brackets. Infectivity of sporozoites was assessed by infected mosquito bite back experiments of C57/BL6 mice at day 21 post haemocoel inoculation. Parasitaemia was monitored for 14 days post mosquito bite. SEM represents the standard error of the mean.
| Description                                                   | Fold enrichment | P value    | Bonferroni correction |
|---------------------------------------------------------------|-----------------|------------|-----------------------|
| **Biological process:**                                       |                 |            |                       |
| Locomotion                                                   | 5.92            | 4.27E-06   | 0.001                 |
| Movement in host environment / symbiotic organism            | 6.39            | 6.94E-06   | 0.002                 |
| Entry into host or symbiotic organism                        | 7.1             | 1.03E-05   | 0.002                 |
| Interaction with host                                        | 5.7             | 1.83E-05   | 0.004                 |
| Multi-organism process                                       | 4.93            | 2.31E-05   | 0.005                 |
| Interspecies interaction between organisms / mutualistic symbionts through parasitism | 5.41            | 2.83E-05   | 0.006                 |
| Entry into host cell / symbiotic cell of another organism    | 7.1             | 3.84E-05   | 0.009                 |
| **Cellular component:**                                      |                 |            |                       |
| Microneme                                                    | 9.56            | 4.61E-06   | 0.004                 |
| Apical part of cell                                          | 4.88            | 9.56E-06   | 0.008                 |
| Apical complex                                               | 4.99            | 5.54E-05   | 0.004                 |
Table S8. *P. falciparum* transmission blocking effect of the α-Pfc43<sup>opt</sup> antibody in SMFA

| Assay   | α-Pfc43<sup>opt</sup> concentration (µg/mL) | Number of midguts | Prevalence (%) | P-value prevalence | Infection Intensity | Oocyst range | P-value intensity |
|---------|-------------------------------------------|-------------------|----------------|-------------------|--------------------|--------------|------------------|
|         |                                           |                   |                |                   | Arithmetic mean (m) | Median (M)   |                  |
| Total   | 0                                         | 137               | 70.8           | 0.2843            | 22.05              | 5.0          | 0-332            | 0.2926            |
|         | 50                                        | 119               | 63.9           | 0.0013            | 13.73              | 4.0          | 0-133            | <0.0001           |
|         | 125                                       | 171               | 44.4           | <0.0001           | 9.45               | 0.0          | 0-250            | <0.0001           |
|         | 250                                       | 171               | 45.6           | <0.0001           | 5.25               | 0.0          | 0-92             | <0.0001           |
| Replicate 1 | 0                                         | 28                | 89.3           |                   | 71.29              | 47.0         | 0-332            | 0.0019            |
|         | 125                                       | 28                | 46.5           | 0.013             | 34.82              | 0.0          | 0-230            | <0.0001           |
|         | 250                                       | 26                | 46.2           | 0.0010            | 8.731              | 0.0          | 0-92             | 0.0586            |
| Replicate 2 | 0                                         | 25                | 68             |                   | 4.56               | 2.0          | 0-18             | 0.0180            |
|         | 125                                       | 40                | 67.5           | 0.1292            | 2.02               | 1.0          | 0-52             | 0.4494            |
|         | 250                                       | 40                | 45.0           | 0.0468            | 1.67               | 0.0          | 0-45             | <0.0001           |
| Replicate 3 | 0                                         | 40                | 55.0           |                   | 7.27               | 2.0          | 0-53             | 0.2105            |
|         | 50                                        | 76                | 57.9           | 0.8444            | 11.64              | 2.0          | 0-133            | 0.0725            |
|         | 125                                       | 49                | 44.9           | 0.3974            | 10.00              | 0.0          | 0-250            | 0.0725            |
|         | 250                                       | 58                | 36.2           | 0.0971            | 4.57               | 0.0          | 0-53             | 0.0725            |
| Replicate 4 | 0                                         | 44                | 75.0           |                   | 16.04              | 8.0          | 0-58             | 0.5907            |
|         | 50                                        | 43                | 74.4           | 1.0000            | 19.52              | 11.5         | 0-83             | <0.0001           |
|         | 125                                       | 54                | 37.0           | 0.0002            | 3.1                | 0.0          | 0-21             | <0.0001           |
|         | 250                                       | 47                | 57.4           | 0.1207            | 9.38               | 2.0          | 0-42             | 0.0185            |

Oocyst data at 7 days post blood feeding from *P. falciparum* SMFAs using the α-Pfc43<sup>opt</sup> antibodies. P values for infection prevalence were calculated using the Fisher’s exact test and P values for infection intensities on the basis of the median number of oocysts was calculated using the Mann-Whitney U test.
| Assay       | Antibody/concentration (µg/mL) | Number of midguts | Prevalence (%) | P-value1 (Prevalence) | Infection Intensity | Oocyst range | P-value2 (Intensity) |
|------------|-------------------------------|-------------------|----------------|-----------------------|---------------------|--------------|---------------------|
|            |                               |                   |                |                       | Arithmetic mean (m) | Median (M)   |                     |
|            |                               |                   |                |                       |                     |              |                     |
| Total      | α-Pbc43/50                    | 110               | 58.2           | 0.0035                | 13.0                | 2.0          | 0.98                | 0.001                |
|            | UPC10/50                      | 126               | 76.2           |                       | 21.9                | 9.0          | 0.172               |                      |
|            | α-Pbc43/100                   | 113               | 52.2           |                       | 6.25                | 1.0          | 0.78                |                      |
|            | UPC10/100                     | 126               | 81.0           | <0.0001               | 22.9                | 14.5         | 0.131               | <0.0001              |
|            | α-Pbc43/250                   | 119               | 27.7           |                       | 2.3                 | 0.0          | 0.27                |                      |
|            | UPC10/250                     | 128               | 80.5           | <0.0001               | 23.7                | 14.0         | 0.189               | <0.0001              |
| Replicate 1| α-Pbc43/50                    | 37                | 64.9           | 0.4059                | 24.3                | 21.0         | 0.98                | 0.0429               |
|            | UPC10/50                      | 26                | 76.9           |                       | 46.3                | 40.0         | 0.172               |                      |
|            | α-Pbc43/100                   | 42                | 50.0           |                       | 8.6                 | 0.5          | 0.78                |                      |
|            | UPC10/100                     | 35                | 94.3           | <0.0001               | 43.3                | 42.0         | 0.131               | <0.0001              |
|            | α-Pbc43/250                   | 32                | 9.4            |                       | 0.50                | 0.0          | 0.8                 |                      |
|            | UPC10/250                     | 45                | 82.2           | <0.0001               | 34.4                | 23.0         | 0.189               | <0.0001              |
| Replicate 2| α-Pbc43/50                    | 35                | 57.1           |                       | 5.1                 | 1.0          | 0.48                | 0.0068               |
|            | UPC10/50                      | 50                | 68.0           | 0.3630                | 19.0                | 10.5         | 0.136               |                      |
|            | α-Pbc43/100                   | 50                | 56.0           |                       | 5.1                 | 1.5          | 0.64                |                      |
|            | UPC10/100                     | 50                | 74.0           | 0.0928                | 17.2                | 12.0         | 0.86                | 0.0004               |
|            | α-Pbc43/250                   | 50                | 38.0           |                       | 3.5                 | 0.0          | 0.22                |                      |
|            | UPC10/250                     | 43                | 79.1           | <0.0001               | 20.8                | 9.0          | 0.162               | <0.0001              |
| Replicate 3| α-Pbc43/50                    | 38                | 52.6           | 0.0020                | 6.5                 | 2.0          | 0.40                | 0.0052               |
|            | UPC10/50                      | 50                | 84.0           |                       | 12.0                | 6.0          | 0.79                |                      |
|            | α-Pbc43/100                   | 21                | 47.6           |                       | 4.3                 | 0.0          | 0.23                |                      |
|            | UPC10/100                     | 41                | 79.0           | 0.0222                | 12.5                | 8.0          | 0.44                | 0.0067               |
|            | α-Pbc43/250                   | 37                | 29.7           |                       | 2.2                 | 0.0          | 0.27                |                      |
|            | UPC10/250                     | 40                | 80.0           | <0.0001               | 14.6                | 7.0          | 0.70                | <0.0001              |

Oocyst data at 10 dpbf from *P. berghei* SMFA’s using the α-Pbc43 antibodies. *P. berghei* SMFA’s with UPC10 antibodies served as a control. P values for infection prevalence were calculated using the Fisher’s exact test and P values for infection intensities on the basis of the median number of oocysts was calculated using the Mann-Whitney U test.
Table S10. Primers for RT-PCR, qRT-PCR, protein expression and generation of transgenic parasites

| Primer name | Sequence (5’ to 3’) | Description |
|-------------|----------------------|-------------|
| *Pfc43* RT-PCR F | GTTGATATAAAAAACAGATGATTTTACTTAAT |  |
| *Pfc43* RT-PCR R | GAAATTTTTATACTAGAATATATGGAAGAACCACACAT |  |
| *Pfs25* RT-PCR F | GCGAAAGTTACGTGGATACTG |  |
| *Pfs25* RT-PCR R | ACTCCAGTTTTAAACAGGATTGCT |  |
| *PICSP* RT-PCR F | TGGCAAGGCTCAATAATGCCA |  |
| *PICSP* RT-PCR R | ACGACATTAAAAACACACTGGAACA |  |
| *Pbc43* RT-PCR F | ATACGGGGAATATCAGAATT |  |
| *Pbc43* RT-PCR R | ACTTCAAAAGCACCCTTGTC |  |
| *Pbs28* RT-PCR F | AATGCACAGGTACAGGAAATATAAT |  |
| *Pbs28* RT-PCR R | CACACTCATATAATGTTCCGCAATT |  |
| *PbCTR* RT-PCR F | AGAGAAGAAGATTTATGGAAGAACCAAAT |  |
| *PbCTR* RT-PCR R | ACTCCAGTTTTAAACAGGATTGCT |  |
| *GFP* RT-PCR F | GCGAAAGTTACCGTGGATACTG |  |
| *GFP* RT-PCR R | ACTCCAGTTTTAAACAGGATTGCT |  |
| *GFP* qRT-PCR F | GCGAAAGTTACCGTGGATACTG |  |
| *GFP* qRT-PCR R | ACTCCAGTTTTAAACAGGATTGCT |  |
| *S7* qRT-PCR F | GTGCCGCGATTTCACAGGAAAGAACCAAAT |  |
| *S7* qRT-PCR R | CACACTCATATAATGTTCCGCAATT |  |
| *LRIM1* qRT-PCR F | AAGTGTTGCAATGCTAATGTTTGGAA |  |
| *LRIM1* qRT-PCR R | CACACTCATATAATGTTCCGCAATT |  |
| *APL1C* qRT-PCR F | GCGATCTCAGGACATCGAATGCT |  |
| *APL1C* qRT-PCR R | GCGATCTCAGGACATCGAATGCT |  |
| *TEP1* qRT-PCR F | AAGTGTTGCAATGCTAATGTTTGGAA |  |
| *TEP1* qRT-PCR R | CACACTCATATAATGTTCCGCAATT |  |
| *Pbc43 opt IF F* | GACAAGCTTTGGCAAGGACACGACCGCAAAAGAC | E. coli expression |
| *Pbc43 opt IF R* | TGCTCGAGTGCAGGGCGCAAGTGGTGTAGACTTGGTCTTGGTAAT | E. coli expression |
| *Pbc43 opt IF F* | GACAAGCTTTGGCAAGGACACGACCGCAAAAGAC | E. coli expression |
| *Pbc43 opt IF R* | TGCTCGAGTGCAGGGCGCAAGTGGTGTAGACTTGGTCTTGGTAAT | E. coli expression |
| *Pbc43 LIC F* | GACAGATCGGAGAGATGATGATGATGATGATG | SF9 expression |
| *Pbc43 LIC R* | GACAGATCGGAGAGATGATGATGATGATGATG | SF9 expression |
| *P1 F* | TGGGCCCCCGAACACTTGGATATGATGATGATGATG | Disruption upstream target Apal |
| *P2 R* | CCAAGCTTTGGCAAGGACACGACCGCAAAAGAC | Disruption upstream target HindIII |
| *P3 F* | TGGGCCCCCGAACACTTGGATATGATGATGATGATG | Disruption downstream target EcoRI |
| *P4 R* | TGGGCCCCCGAACACTTGGATATGATGATGATGATG | Disruption downstream target BamHI |
| INT F1 (P5) | GATCGAAATAAACAAATTGGGAATAG | Diagnostic primer WT and KO/c507 |
| Primer Code | Sequence | Description |
|-------------|----------|-------------|
| WT R (P6)   | GTACTACCTCACCCTGTATTACCAG | Diagnostic primer WT/c507 |
| TgDHFR 5'UTR R (P7) | GATGTGGTTATGTAATGCATACAC | Diagnostic primer KO/c507 |
| PlasmoGEM QCR1 F(P8) | AGGAATTGTTAGTTTTTTGTTTTG | Diagnostic primer WT/1804c1 |
| PlasmoGEM QCR2 R(P9) | CTGCTACACTTATGTCGCC | Diagnostic primer WT/1804c1 |
| PlasmoGEM GW1 F(P10) | CGGGGCCCTATGCAATATC | Diagnostic primer KO/1804c1 |
| PlasmoGEM GT R (P11) | GTGCACGTGCTTGAGTGG | Diagnostic primer KO/1804c1 |
| P12 F      | CCAAGCTTGGTTAATGATAATACATGGATATATG | Pfc43 complement upstream target HindIII |
| P13 R      | GGGGGGCTATCTGTTTTTTATCTTTGAAAATAAAA | Pfc43 complement upstream target Apal |
| P14 F      | TTGGGGCAGTTGCAAGAAACGAATTATGATCATATT | Pfc43 CDS Apal |
| P15 R      | GGGGCGGTTAATATGTATATGATAACAAAAACATTAAT | Pfc43 CDS SacII |
| P16 F      | GGCAGGCTCATATGTAAGGACGAAACATATTAGTGTATTAT | Pbf43 3'UTR for Pfc43 complement SacII |
| P17 R      | GGCAGGTCTATAACCTATTTTCTATTATTGGAAT | Pbf43 3'UTR for Pfc43 complement SacII |
| P18 F      | TTCTCGAGGAGCTGGAATTACGACAAAAAGGTTGTTTG | Pfc43 complement downstream target XhoI |
| P19 R      | TTCCGAGGTTTCTAATAATTATTTGATGAAAA | Pfc43 complement downstream target SmaI |
| INT F2 (P20) | GGATCTTTGGATGCTTCTTTCCCTAAGAAG | Diagnostic primer WT, Pfc43 and Pbc43 comp |
| Pfc43 INT R (P21) | TCCTCTCGGTACAAGTGGCCCATATCCTTTT | Diagnostic primer Pfc43 complement |
| P22 F      | TTAAGCTGAGGCGCCCTTCTTATGGTGTAAAAG | Pbc43 WT complement upstream target HindIII |
| P23 R      | GGCAGGCTCATATGTAAGGACGAAACATATTAGTGTATTAT | Pbc43 WT complement upstream target SacII |
| P24 F      | TTCTCGAGTAAATATGTATATGAAAGCAGAGATT | Pbc43 WT complement downstream target XhoI |
| P25 R      | TTCCGAGGTTTCTAATAACTTATTTCATATT | Pbc43 WT complement downstream target SmaI |
| Pbc43 INT R (P26) | AACATATATAAACATAGATGCTACTTTTAC | Diagnostic primer Pbc43 WT complement |

Where appropriate, target restriction sites are shown as underlined italics and restriction site overhangs are also shown. The appropriate restriction enzyme is presented in the description column. F, forward; R, reverse; LIC, ligation independent cloning; IF, in-fusion; INT, integration; WT, wild-type; KO, knockout; UTR, untranslated region. All primers are listed in a 5' to 3' direction.
Supplementary materials and methods

Sequence analysis
Plasmodium DNA and protein sequences were retrieved from PlasmoDB (http://plasmodb.org/plasmo/) and aligned using ClustalW2 in the BioEdit sequence alignment editor program. Signal peptide and transmembrane domains were predicted using SignalP4.0 (1) and TMHMM Server v. 2.0 (2), respectively.

**P. berghei** strains and culturing

P. berghei ANKA lines used include: cl15cy1 (2.34), the reference parent line of P. berghei ANKA, 507m6cl1 (c507) constitutively expressing GFP integrated into the 230p gene locus (PBANKA_0306000) without a drug selectable marker (3), 1804cl1 (c1804) constitutively expressing mCHERRY integrated into the 230p locus without a drug selectable marker (4), and 2.33 (5), a non-gametocyte producer line. All lines were maintained in 8-10 week old CD1 and/or Balb/c female mice by serial passaging. Culturing and purification of P. berghei ABS, gametocytes and ookinetics were carried out as described previously (3).

**P. falciparum** culturing

P. falciparum NF54 was cultured as described previously (6). Briefly, ABS and gametocytes were cultured using hRBCs of various blood groups in the following order of preference: O+ male, O+ female, A+ male and A+ female. Set up of PNHF54 asexual culture was performed in T25 cm² flasks containing a final volume of 500 μL of hRBCs (0.3-4% infection) and 10 mL of CM RPMI-1640-R5886 (Sigma), 0.05 g/L Hypoxanthine, 0.3 mg/L L-glutamine powder (G8540-25G Sigma) and 10% (v/v) sterile human serum of A+ serotype), kept at 37°C incubation and supplemented with “malaria gas” [3% O₂/5% CO₂/92% N₂ (BOC Special Gases, cat. no. 226957-L-C)]. Gametocyte cultures were initiated by diluting the continuous asexual culture (3-4% ring forms) to 1% ring forms by the supply of fresh hRBCs. Gametocyte cultures were kept at constant temperature of 37°C until day 14 ensuring daily exchange of around 75% of the medium per flask.

RT-PCR and quantitative RT-PCR

Total RNA was extracted from P. falciparum and P. berghei parasites using Trizol reagent (ThermoFisher) according to the manufacturer's instructions. cDNA was synthesized using the Primerscript Reverse Transcription Kit (Takara) after Turbo DNase (ThermoFisher) treatment. For RT-PCR, the resulting cDNA was used in the PCR of P. falciparum and P. berghei PbPIMMS43 using gene specific primers (Table S10). P. falciparum Pls25 and PICS, and P. berghei P28 and CTRP served as stage specific controls. Constitutively expressed GFP in P. berghei was used as an internal control. For qRT-PCR, SYBR green (Takara) was used according to the manufacturer's guidelines. Expression of PbPIMMS43 was normalized against GFP and expression of TEP1, LRIM1 and APL1C was normalized against S7 using the ∆ΔCt method.

Expression and purification of recombinant PbPIMMS43 in E. coli

PbPIMMS43 and PbPIMMS43 was codon optimized for expression in E. coli (GeneArt, ThermoFisher) and termed Ptc43opt and Pbc43opt, respectively. Ptc43opt (aa 25-481) and Pbc43opt (aa 22-327) fragments that both exclude the signal peptide and the C-terminal hydrophobic domain were amplified with primers (Table S10) and cloned into a NotI digested protein expression vector plasmid, pET-32b (which carries N and C-terminal 6xHistidine tags) (Novagen) by In-Fusion Cloning (Takara). Shuffle T7 E. coli cells (NEB) containing the recombinant protein expression plasmid were grown at 30°C and induced with 1 mM isopropyl-1-thio-β-d-galactopyranoside at 19°C for 16 h. Cells were harvested and lysed using bugbuster-lysonase (Novagen) containing protease inhibitors (cComplete EDTA-free, Roche). Cell debris were removed by centrifugation. Both proteins were expressed as His-fusion proteins and hence purified by cobalt affinity chromatography using TALON® metal affinity resin (Takara). The Pbc43opt recombinant protein was soluble and purified under native conditions in phosphate buffered saline (PBS), pH 7.4. The Pbc43opt recombinant protein was extracted from inclusion bodies using the Inclusion Body Solubilization Reagent (ThermoFisher). The solubilized protein was then purified under denaturing conditions in 8M urea in PBS, pH 7.4. Refolding of Pbc43opt was carried out in decreasing concentrations of urea in PBS. Protein samples were analyzed by SDS-PAGE to determine purity prior to their use for immunization in rabbits for the generation of the affinity purified polyclonal antibodies α-Ptc43opt and α-Pbc43opt (Eurogentec).

Expression and purification of recombinant PbPIMMS43 in Sf9 cells

PbPIMMS43 (aa 22-331) that excludes the signal peptide and includes four amino acids of the C-terminal hydrophobic domain was amplified from 24 h *in vitro* ookinetes cDNA using primers in Table S10. This fragment was cloned by ligation independent cloning into the linearized pIX-10 EK-LIC vector which carries a C-terminal 10xHis tag (Novagen) to generate pEX-10: Pbc43-SP/TM. A stable line expressing the
recombinant protein was generated by co-transfection of pIEX-10: Pbc43-SP/TM and pIEX-10:Neo plasmid(7) using the Cellfectin® II Reagent (ThermoFisher) according to the manufacturers’ guidelines. pIEX-10:Neo plasmid carries the neomycin resistance cassette and provides resistance to the antibiotic G418 (Sigma). The recombinant protein was extracted from cells using lysis buffer (1XPBS, 1% v/v Triton X-100, pH 7.4) containing benzonase (Novagen) and protease inhibitors. The His-tagged recombinant PbPIMMS43 protein was insoluble and extracted by solubilization in 8M urea in PBS, pH 7.4. The protein was purified using TALON® metal affinity resin under denaturing conditions in 8M urea in PBS, pH 7.4. Bound proteins were eluted using denaturing elution buffer. Refolding of Pbc43 was carried out in decreasing concentrations of urea in PBS. Protein samples were analyzed by SDS-PAGE to determine purity prior to their use for immunization in rabbits in the generation of the affinity purified polyclonal antibody α-Pbc43S99 (Eurogentec).

Western blot analysis
Whole cell lysates were prepared by suspending purified parasite pellets in whole cell lysis buffer (1XPBS, 1% v/v Triton X-100) containing protease inhibitors. Using the previously described method (28), 24 h in vitro ookinetes were subjected to cellular fractionation to obtain the soluble, Triton-Soluble and insoluble cell fractions. Protein samples were then boiled under non-reducing or reducing (3-5% v/v 2-mercaptoethanol) conditions in Laemilli buffer and separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Separated proteins were then transferred to a PVDF membrane (GE Healthcare). Proteins were detected using α-Pbc43S99 (1:100), α-Pfc43S99 (1:100), goat α-GFP (Rockland chemicals) (1:100) and 13.1 mouse monoclonal α-P28 (8) (1:1000) antibodies. Secondary horseradish peroxidase (HRP) conjugated goat α-rabbit IgG, goat α-mouse IgG antibodies (Promega) and donkey α-goat IgG (Abcam) were used at 1: 10,000, 1: 10,000 and 1: 5,000 dilutions, respectively. All primary and secondary antibodies were diluted in 3% milk-PBS-Tween (0.05% v/v) blocking buffer.

Indirect immunofluorescence assay
IFA's were carried out on blood bolus parasites, ookinetes invading the midgut epithelium, oocysts, oocyst sporozoites, and salivary gland sporozoites. For IFA's on blood bolus parasites, midguts of blood fed mosquitoes at 1 hpf were dissected, and the blood boluses were collected. Blood bolus was washed in PBS prior to fixation in 4% paraformaldehyde (PFA) in PBS for 30 min. Fixed parasites were smeared on glass slides, allowed to air dry, permeabilized with 0.2% v/v Triton X-100, and blocked in a 3% w/v bovine serum albumin (all diluted in PBS). For IFA's on ookinetes invading the midgut epithelium at 24-30 hpf, or young oocysts at 2 dpbf, midguts of blood fed mosquitoes were dissected, and blood boluses were discarded. The midgut epithelium was fixed in 4% PFA in PBS for 45 min and washed thrice in PBS for 10 min each. Midgut epithelium was permeabilized and blocked for 1 h with 1% w/v BSA, 0.1% v/v Triton X-100 in PBS. For IFA's on sporozoites, infected midguts and salivary glands were dissected, and tissues were homogenized to release sporozoites. Sporozoites were fixed, blocked and permeabilized as that used for blood bolus parasites. Samples were then stained in blocking solution with primary antibodies (α-Pfc43S99, 1:300; 4B7 mouse monoclonal α-Pfs25 (9), 1:1000; 2A10 mouse monoclonal α-PfCSP (10), 1:200; α-Pbc43S99, 1:100; 13.1 mouse monoclonal α-P28, 1:1000; and 3D11 mouse monoclonal α-PbCSP (11), 1:100. Alexa Fluor (488 and 568) conjugated secondary goat antibodies specific to rabbit or mouse (ThermoFisher) were used at a dilution of 1:1000. 4',6-diamidino-2-phenylindole (DAPI) was used to stain nuclear DNA. Images were acquired using a Leica SP5 MP confocal laser-scanning microscope. Images underwent processing by deconvolution using Huygens software and were visualized using Image J.

Generation of transgenic parasites
Partial ko of P. berghei c43 CDS was carried out by double crossover homologous recombination in the c507 and 1804cl1 lines. For partial disruption in the c507 line, a 765 bp Apal/HindIII 5' homology arm and a 528 bp EcoRI/BamHI 3' homology arm was amplified from P. berghei 2.34 genomic DNA using the primer pairs P1/P2 and P3/P4 respectively. These fragments were cloned into the pBS-TgDHFR vector which carries a modified Toxoplasma gondii dihydrofolate gene (TgDHFR/TS) cassette that confers resistance to pyrimethamine(12). The targeting cassette was released by Apal/BamHI digestion and it allows ko of 50% of P. berghei c43 CDS at the 5' region. For partial disruption in the 1804cl1 line, the target vector containing the human DHFR selection cassette was used (kindly provided by plasmoGEM, vector design ID, PbGEM-042760; http://plasmogem.sanger.ac.uk/). hDHFR confers resistance to the drugs pyrimethamine and WR99210. The targeting cassette was released by NotI digestion and allows ko of 74% of PbPIMMS43 CDS leaving a small part of the 3' region of the CDS.

To express P. falciparum c43 in P. berghei, the transgenic parasite PbPc43 was created in the c507 line. The Ppc43 replacement construct was generated using the plasmid pL0035 which carries the hDHFR selection cassette (13). A 1.7 kb HindIII/Apal fragment upstream of the PbPIMMS43 coding DNA sequence was amplified using primer pair P12/P13 and cloned upstream of the pL0035 selectable marker cassette. The 1.5
kb Plc43 CDS was amplified from cDNA using the Apal and Saccl primer pair P14/P15 and cloned downstream of the 1.7kb region. A 518 bp region corresponding to the 3'UTR, downstream of the PbPIMMS43 stop codon, was amplified as a Saccl fragment using primers P16/P17 and cloned downstream of the 1.5 kb Plc43 CDS. Downstream of the l0035 selectable marker, a 700 bp region corresponding to part of the PbPIMMS43 coding region and part of the 3'UTR was amplified using primers P18/P19 and cloned as a Xhol and Smal fragment. This replacement vector was linearized by HindIII and Smal digestion. To re-introduce PbPIMMS43 into the Δc43 ko parasite, the transgenic parasite Δc43::c43 was created. A 3.5 kb upstream region that includes the PbPIMMS43 ORF and its 5'UTR and 3'UTR was amplified as a HindIII and Saccl fragment using primers P22/P23. A 518 bp downstream region corresponding to the PbPIMMS43 3'UTR was amplified as a Xhol and Smal fragment using primers P24/P25. These fragments were cloned into the pL0035 vector and served as homology regions for homologous recombination at the Δc43 ko locus in the c507 line. This replacement vector was linearized by HindIII and Smal digestion. Transfection of linearized constructs, selection of transgenic parasites and clonal selection was carried out as described previously (3).

Genotypic analysis of transgenic parasites
Purified blood stage parasites were obtained after white blood cells removal using hand packed cellulose (Sigma) columns and red blood cell lysis in 0.17M NH4Cl on ice for 20 min. Genomic DNA was extracted from parasites using the DNeasy kit (Qiagen). Detection of successful integration events or maintenance of the unmodified locus was performed by PCR on genomic DNA using primers listed in Table S10. Blood stage parasites within agarose plugs were lysed in lysis buffer (1XTNE, 0.1 M EDTA pH 8.0, 2% (v/v) Sarkosyl, 400μg/mL protease K) to release nuclear chromosomes. Southern blot analysis on pulsed field gel electrophoresis separated chromosomes (Run settings: 98 volts, 1-5 mins pulse time for 60 h at 14 °C) was carried out with a probe targeting the TgDHFR/TS-P. berghei DHFR 3'UTR, obtained by HindIII and EcoRV digestion of the pBS-TgDHFR plasmid.

Phenotypic assays
Exflagellation assays were performed as described previously (14). Briefly blood from a high gametocytemia mouse was added in a 1:40 ratio to oocyte medium (RPMI 1640, 20% v/v FBS, 100 μM xanthurenic acid, pH 7.4), and exflagellation was counted in a standard haemocytometer under a light microscope. In vitro conversion assays were performed as previously described (14). For in vivo conversion assays, the blood bolus of 10 mosquitoes at 17-18 hpf was pelleted, washed in PBS and resuspended in 50 μL of fresh oocyte medium. The suspension was then incubated with a Cy3-labelled 13.1 mouse monoclonal α-P28 (1:50 dilution) for 20 min on ice. The conversion rate was calculated as the percentage of Cy3 positive ookinetes to Cy3 positive macrogametates and ookinetes.

Ookinet motility assays were performed as described (15). Briefly, 24 h in vitro ookinete culture was added to Matrigel (BD biosciences), dropped onto a slide, sealed with nail varnish, and allowed to set at RT for 30 min. Time-lapse microscopy (1 frame every 5 seconds, for 10 min) of ookinetes were taken on a Leica DMR fluorescence microscope and a Zeiss Axiocam HRc camera controlled by the Axiovision (Zeiss) software. The speed of individual ookinetes was measured using the manual tracking plugin in the Icy software package (http://icy.bioimageanalysis.org/).

Ookinite injections in mosquito haemocoel
24 h in vitro ookinetes was adjusted with RPMI 1640 to achieve an injection concentration of 800 ookinetes per mosquito as described previously (16). This was injected into the thorax of A. coluzzii mosquitoes using glass capillary needles and the Nanoject II microinjector. Salivary gland sporozoites were counted at 21 dpbf as described below.

Gene silencing
cDNA was prepared from total RNA extracted (as described above) from A. coluzzii midgut infected with P. berghei c507, at 24 hpf. The cDNA was used in the amplification of CTL4, LRIMI and TEPI using primers with T7 overhangs as reported in (17, 18). The resulting T7 PCR products and the T7 high yield transcription kit (ThermoFisher) was used to produce dsRNA. DsRNA was purified using the RNeasy kit (Qiagen) and 0.2 μg in 69 nL was injected into the thorax of A. coluzzii mosquitoes using glass capillary needles and the Nanoject II microinjector (Drummond Scientific). Injected mosquitoes were left for 2-3 days before P. berghei infection.

RNA-sequencing library preparation
Three replicate infections of A. coluzzii mosquitoes with the Δc43 and c507 P. berghei lines were performed and infected midguts were dissected at 1 and 24 hpf. Total RNA was extracted as described elsewhere and was used for RNA sequencing by Genewiz (New Jersey, US) using the NEB Ultra prep kit and an Illumina
HiSeq platform with 150x2 paired-end reads. Prior to the RNA sequencing, successful infection of the midgut epithelium was confirmed by P28-staining of parasites in 5 midguts from each replicate infection: Replicate 1, c507 median 536 (458, 635, 495, 536, 598), Δc43 median 501 (419, 436, 501, 605, 520), Replicate 2, c507 median 386 (386, 421, 350, 258, 408), Δc43 median 389 (347, 411, 389, 369, 402) and Replicate 3, c507 median 548 (501, 426, 548, 603, 551), Δc43 median 495 (495, 504, 521, 465, 436).

NGS RNA-sequencing—Data processing and analysis
RNA-Seq reads were mapped using HiSat2 v2.0.5 (19) with default parameters to the A. gambiae genome (AgamP4 assembly) (20) and the P. berghei ANKA (21). Transcript abundance was quantified as fragments per kilobase per million reads (FPKM) using Cufflinks v2.2.1 (22) on the A. gambiae (Anopheles-gambiae-PEST_BASEFEATURES_AgamP4.9.gtf) and P. berghei (PlasmoDB-39_PbergheiANKA.gff) annotation sets. Differential expression analysis was performed using Cuffdiff v.2.2.1 (23). The sequencing data were uploaded to the Galaxy web platform (an open source, web-based platform for data intensive biomedical research), and we used the VectorBase Galaxy server (https://galaxy.vectorbase.org) to analyze the data (24). Data are derived from three independent biological replicates, each of which included three technical replicates. To filter out the biological or technical noise from the actively expressed genes, an FPKM cutoff was selected that is based on an implementation of the zFPKM normalization method described previously (25). Functional classification of P. berghei differential regulated genes were performed in PlasmoDB (http://plasmodb.org/plasmo/) using the P. berghei full genome as a reference genome. PANTHER (v13.1; http://pantherdb.org) (26) was used for functional classification of A. gambiae differentially regulated genes. The RNA sequencing data were deposited to and can be downloaded from the European Nucleotide Archive with experiment codes ERX3197375-410.

Population genetics analysis
The genome sequences of 1,509 African P. falciparum samples determined in the context of the P. falciparum Community Project were obtained from the MalariaGen website (http://www.malariagen.net/data). They include samples from 11 African countries including Gambia (73), Guinea (124), Mali (87), Burkina Faso (56), Ghana (478), DR of the Congo (279), Uganda (12), Tanzania (68), Malawi (262) and Madagascar (18). Call of SNPs found in A. gambiae samples determined in the context of the Population Genetics Analysis of the Human Population of Africa (Community Project) were obtained from the MalariaGen website (http://www.malariagen.net/data). Population genetics analysis was performed using the R (v.3.2.1) packages gdsfmt and SNPRelate (27) by considering (a) all SNPs across each gene and all populations within a given country and (b) each individual SNP sampled from populations in each of the 11 African countries (FST total) and in pairwise country comparisons.

P. berghei mosquito infections
Mosquitoes were infected with P. berghei by utilizing direct feeding assays (DFAs) or standard membrane feeding assays (SMFAs). DFAs were carried out by mosquito feeding on mice with 5-6% parasitaemia and 1-2% gametocytaemia. SMFAs were carried out as described previously (28). Briefly, for each feed, 350 μL of heparanized P. berghei ANKA 2.34 infected blood from mice with 5-6% parasitaemia and 2-3% gametocytaemia was mixed with 150 μL of PBS containing either α-Pbc43SR or the isotopic monoclonal UPC10 (negative control; Sigma) antibodies to yield final antibody concentrations of 50, 100 and 250 μg/mL. Infected mosquitoes were maintained at 19-21°C, 70-80% humidity, 5% sucrose and 12/12 hours light/dark cycle.

Midguts tissues were dissected at 4-10 dpbf and fixed in 4% PFA in PBS. Fixed midguts were mounted in Vectashield® (VectorLabs) and oocysts or melanised ookinetes were enumerated using light and/or fluorescence microscopy. Oocyst images and sizes were also analyzed using fluorescence microscopy. Oocyst and salivary gland sporozoite numbers at 15 and 21 dpbf respectively were counted using a standard haemocytometer, in 3 technical replicates of homogenates of 10 P. berghei infected A. coluzzii midguts or salivary glands.

Finally, in mosquito to mouse transmission assays, at least 30 P. berghei infected mosquitoes were allowed to feed on 2-3 anaesthetized C57BL/6 mice at 20-22 dpbf. Parasitaemia was monitored up until 14 days post mosquito bite by Giemsa stained tail blood smears.

P. falciparum mosquito infections
Mosquitoes were infected with P. falciparum by SMFAs as described previously (6). Briefly, gametocytemia and density of viable mature stage V gametocytes at day 14 post-induction were assessed by Giemsa stained blood smears and by testing in vitro exflagellation of male gametocytes respectively. Day 14 stage V gametocytes cultures were pooled in a pre-warmed tube containing 20% v/v uninfected serum-free hRBCs and 50% v/v heat-inactivated human serum to a final volume of 300 μL. The mixture was immediately transferred to pre-warmed glass feeders kept at a constant temperature of 37°C and mosquitoes were allowed to feed. For transmission blocking assays, α-Pfc43SR antibodies were added to the gametocytemic blood mix
to final antibody concentrations of 50, 125 and 250 µg/mL, in a final volume of 300 µL. Blood fed mosquitoes were maintained at 27°C, 70% humidity, 5% sucrose and 12/12 hours light/dark cycle. Infected midgut tissues at 10 dpbf were dissected and analyzed as above.

Statistical analysis
Statistical analyses for exflagellation, ookinete conversion and motility assays were performed using a two-tailed, unpaired Student’s t-test. For statistical analyses of the oocyst or melanized parasite load (infection intensity) and presence of oocysts (infection prevalence), p values were calculated using the Mann-Whitney test and the Fisher’s exact test, respectively. Statistical analyses were performed using GraphPad Prism v7.0 and v8.0. The generalized linear mixed model (GLMM) was used to also determine statistical significance in oocyst infection intensity in transmission blocking assays. GLMM analyses were performed in R (version 2.15.3) using the Wald Z-test on a zero-inflated negative binomial regression (glmmADMB). The various treatments were considered as covariates and the replicates as a random component. Fixed effect estimates are the regression coefficients.
Supplementary Dataset legends

Dataset S1 (separate file). RNAseq transcription profiling of *P. berghei* Δc43 and c507 lines in the *A. coluzzii* midgut
Transcription profiles are assessed at (A) 1 hpbf and (B) 24 hpbf. FPKM is transcript abundance quantified as fragments per kilobase per million reads in each of the Δc43 and c507 parasite lines. The log2-transformed ratio of Δc43 to c507 FPKM data for each gene is shown (log2 (Δc43/c507)). P values (P value), adjusted P values (q value) and statistical significance (q<0.005; yes or no) are indicated. Data are derived from three independent biological replicates, each of which included three technical replicates.

Dataset S2 (separate file). RNAseq transcription profiling of *A. coluzzii* midgut responses to *P. berghei* Δc43 and c507 infections
Transcription profiles are assessed at (A) 1 hpbf and (B) 24 hpbf. FPKM is transcript abundance quantified as fragments per kilobase per million reads in each of the *A. coluzzii* genes infected with Δc43 (Ac Δc43) and c507 (Ac c507) parasite lines. The log2-transformed ratio of FPKM values for each *A. coluzzii* gene in infections with Δc43 to infections with c507 is shown (log2 (Ac Δc43/Ac c507)). P values (P value), adjusted P values (q value) and statistical significance (q<0.005; yes or no) are indicated. Data are derived from three independent biological replicates, each of which included three technical replicates.

Dataset S3 (separate file). Fixation index analysis of *P. falciparum* PIMMS43 and Pfs47
Fixation index (*F*<sub>ST</sub>) values for all SNPs in (A) PIPIMMS43 and (B) Pfs47 genes are calculated from 1,509 parasites samples from 11 African countries. *F*<sub>ST</sub> values from the analysis of all samples together (*F*<sub>ST</sub> total) and from country pairwise comparisons are shown. NA indicates monomorphic sites. SNP IDs and their position, reference and alternative alleles, and amino acid changes are indicated.
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