Supporting information

Supplementary Figure 1

Generation of two *P. berghei* K173 reporter lines that express the fusion protein GFP-Luciferase under the control of the *ama-1* promoter.

A. The construct (pL0028) for generation of transgenic line K173*cl1-GFP-Luc*$_{schiz\text{-}a}$ that expresses GFP-Luciferase under the control of the *ama1*-promoter and the 3'-UTR of the *dhfr/ts* gene. The construct contains the *tgdhfr* selectable marker cassette (SM) and a target region of the *c/d-small subunit (ssu) rrna* unit for integration by single cross-over homologous recombination.

B. The construct (pL1403) for generation of transgenic line *K173*cl1-*GFP-Luc*$_{schiz\text{-}b}$ that expresses GFP-Luciferase under the control of the *ama1*-promoter and the 3'-UTR of the *dhfr/ts* gene. The construct contains no selectable marker cassette (SM) and two target regions of the *c/d-small subunit (ssu) rrna* unit for integration by double cross-over homologous recombination.

C. Southern analysis of separated chromosome of cloned lines of K173*cl1-GFP-Luc*$_{schiz\text{-}a}$ and K173*cl1-GFP-Luc*$_{schiz\text{-}b}$ confirming correct integration of construct pL0028 and pL1403 into the *c/d-small subunit (ssu) rrna* locus on chromosome 5/6.

D. Representative distribution of non-sequestered schizonts in K173*cl1*-infected mice showing distribution throughout the body as shown by luciferase activity in the upper body (lungs, head), decreased sequestration in adipose tissue and increased accumulation in the spleen (see also Fig. S2).

Supplementary Figure S2

Schematic representation of constructs for generation of transgenic parasites expressing tagged proteins and constructs for gene deletion/disruption

A) Schematic representation of the DNA construct used for generation of transgenic lines expressing proteins C-terminally tagged with mCherry- or eGFP. The construct, which is derived from pL1419

(upper panel), contains the *tgdhfr* selectable marker cassette (SM) and a region of the target gene for integration (black) in the endogenous gene (middle panel) by single cross-over homologous recombination. Integration of the construct into the target gene results in a C-terminal *mCherry/GFP* tagged copy of the target gene (lower panel). Restriction site (RS) used for linearization of the construct refer to Table S6A.

B) Schematic representation of the DNA construct used to disrupt the selected genes shown in Table S4. The construct is aimed at disruption of the target gene by double cross-over homologous recombination and is based on the existing gene-deletion constructs pL0001 and pL0037. Plasmid pL0001 contains the *tgdhfr* selectable marker cassette (SM) and pL0037 contains the h*dhfr*::y*fcu* selectable marker cassette (SM).The sequence of the 5'- and 3' forward (F) and reverse (R) primers to amplify the target regions of the genes are shown in Table S6. All details of the DNA constructs, primers and genotype analyses of the mutants are available in the RMgmDB database of genetically modified malaria parasites (www.pberghei.eu).

C) Schematic representation of PCR-amplified DNA construct used to disrupt 4 of the selected gene listed in TableS4. These constructs contains the h*dhfr* selectable maker (SM; white box) flanked by anchoring primer sequences (hatched boxes) and the recombination sequence targets (grey boxes). The sequence of the 5'- and 3' forward (F) and reverse (R) pL0040-anchoring primers to amplify the target regions of the genes and subsequently the SM are shown in Table S6. All details of the DNA constructs, primers and genotype analyses of the mutants are available in the RMgmDB database of genetically modified malaria parasites (www.pberghei.eu). As a loading control (ctrl) for wild type (wt) parasites we used the aspecific reaction of the antibodies with a ~20 kDa parasite protein.

Supplementary Figure S3

mCherry-tagged proteins with a predominant localization in the cytoplasm of the parasite with no evidence for export into the host erythrocyte

Localization of different *P. berghei* proteins as determined by fluorescence microscopy of mCherry-tagged proteins (red) in live, infected red blood cells (irbc). For most transgenic lines a trophozoite and schizont is shown. Analysis of many blood stages showed a predominant localization in the cytoplasm of trophozoites and schizonts. PBANKA_140060 (line 1915) which is the ortholog of the *P. falciparum* cytoadherence linked asexual protein 2/3 (CLAG2/3) shows a rhoptry-like localization pattern in merozoites. The irbc surface membrane is stained with TER119 antibody (green) and parasite nuclei are stained with Hoechst (blue).

Supplementary Figure S4:

Analysis of expression of mCherry/eGFP-tagged candidates.

Localization of mCherry or eGFP in live *P. berghei* ANKA infected red blood cells of different tagged candidates presented in Figure 2 (different members of three protein-families), Figure 3 (different proteins encoded by single-copy genes), Figure 4 (EMAP1, PBANKA_083680) and Figure 5 (EMAP2, PBANKA_021550).

Parasite nuclei are stained with Hoechst (blue); BF: brightfield.

Supplementary Figure S5:

Analysis of expression of mCherry-tagged PBANKA_136550 and schizont sequestration of a mutant lacking expression of PBANKA_136550 (Δ*pbanka_136550*).

A) Schematic of mCherry-tagged PBANKA_136550 showing the location of PEXEL motif and trans-membrane domain (TM).

B) Analysis of *pbanka_136550::mCherry* parasites (in ANKA-GFP-Luc$_{sch}$ background) Left panel: Southern analysis of separated chromosomes shows integration of the tagging-construct into chromosome 13. Middle panel: Western analysis of PBANKA_136550::mCherry expression using anti-mCherry antibodies. As a loading control (ctrl) for wt parasites we used HSP70 antibodies. Right panel: FACS analysis of mCherry-expressing schizonts. Infected red blood cells (irbc) are selected based on Hoechst- and mCherry-fluorescence. An average percentage of 95% (± 1; n= 2; Gate 2) of the total number of schizonts (Gate 1) is mCherry positive. Gate 1 (G1): mature schizonts (8-16N); Gate 2: mCherry-positive schizonts).

C) A) Localisation of mCherry-tagged PBANKA_136550 in ANKA*wt* blood stages showing a punctate, vesicle like localization in the irbc cytoplasm. Parasite nuclei are stained with Hoechst (blue).

D) Schizont sequestration of Δ*pbanka_136550* parasites. Left panel: Southern analysis of separated chromosomes shows integration of the gene-deletion construct in chromosome 13. Northern analysis shows absence of *pbanka_136550* transcripts (using a PCR-amplified *pbanka_136550* probe); as a loading control (ctrl) for wild type (wt) parasites ethidium bromide stained parasite RNA. Middle panel: Representative tissue distribution of sequestered schizonts in mice infected Δ*pbanka_136550* parasites showing the characteristic CD36-mediated schizont-distribution in adipose tissue (belly) and lungs comparable to the sequestering ANKA-GFP-Luc$_{schiz}$ (see also Fig. S2). Lower panel: FACS analysis of the presence of schizonts in the peripheral blood circulation in mice (n=4) with asynchronous infections of *pbanka_136550.* Tail blood of infected with parasites expressing GFP-Luciferase (under the *ama-1* promoter) is stained with Hoechst and analysed for Hoechst- and GFP-fluorescence. The total number of schizonts (Gate G1: parasites with >2N DNA content) and mature schizonts (Gate G2: parasites expressing GFP) is compared to those in tail blood from mice infected with the non-sequestering

parasites of line K173cl1-GFP-Luc$_{sch-a}$ Both the total number of schizonts and the number of mature schizonts are significantly lower ($p<0.0001$) compared to K173cl1-GFP-Luc$_{sch-a}$.

Supplementary Table S1:

Lists of proteins identified by proteome analyses of proteins associated with membranes of red blood cells infected with P. berghei ANKA, P. berghei K173 and Δsmac.

Supplementary Table S2:

Lists of predicted exported proteins of P. berghei based on published bioinformatics and experimental analyses

Supplementary Table S3:

Characteristics of P. berghei proteins selected for analysis by tagging and characteristics of the mutants expressing the tagged proteins

Supplementary Table S4:

Characteristics of P. berghei proteins selected for functional analysis by gene deletion and characteristics of the gene deletion mutants

Supplementary Table S5:

Genome sequence of K173cl1: Lists of K173cl1 genes that show (partial) deletions, single nucleotide polymorphisms (SNPs) and indels.

Supplementary Table S6:

Sequence and other details of primers used in this study for generation of DNA constructs (gene-tagging and gene-deletion constructs) and sequence of other primers used for making probes, intermediate constructs and constructs for EMAP1 protein expression.