Critical role for a stage-specific actin in male exflagellation of the malaria parasite

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Introduction

Development of male gametes in Plasmodium parasites is composed of two phases [for recent reviews see (Dixon et al., 2008; Sinden, 2009; Kuehn and Pradel, 2010; Sinden et al., 2010)]. The first, gametocytogenesis, takes place in the blood of the vertebrate host, while the second, called gametogenesis, initiates once the blood has been taken up by the mosquito vector. Although these processes are largely similar in different species of Plasmodium there are also fundamental differences (Janse and Waters, 2004). In the human parasite Plasmodium falciparum, gametocyte development is initiated in the ‘parental’ schizont and gametocyte development takes about 7–8 days (Janse and Waters, 2004). In the rodent parasite Plasmodium berghei, used in this study, gametocytes can first be distinguished from the asexual trophozoites by morphological and molecular criteria 15–16 h after invasion of the red blood cell (RBC) by the merozoite. Examples of molecular markers for early sexual development are the pbs230 and mdv-1/peg3 gene transcripts (Janse et al., 2003; Ponzi et al., 2009) and the mRNA from the SET distal promoter (Pace et al., 2006). After another 10 h, the male and female gametocytes are fully matured and, arrested in their cell cycle, circulate in the blood.

Gametogenesis ensues after uptake by the mosquito and is activated by a drop in temperature and the presence of xanthurenic acid (Billker et al., 1997; 1998). A detailed transmission electron microscopy (TEM) study described the process (Sinden et al., 1976). The activation of the female, at least morphologically, is limited to the egress of the parasite from the host cell by disruption of the parasitophorous vacuole (PV) membrane and the RBC membrane. On the other hand, the male undergoes a dramatic process called exflagellation. During a 10 min interval the PV and RBC membranes are disrupted, DNA is replicated three times and eight axonemes, critical components of the motile flagellar gametes, are assembled. These events give rise to rapidly moving male gametes, which fuse with female cells, resulting in the formation of zygotes. At present, we do not have a detailed view of the molecular players of these processes although high-throughput analyses have identified a number of gametocyte-expressed genes in P. falciparum and P. berghei [reviewed in (Kooij and Matuschewski, 2007)].
Actin is a cytoskeletal protein with many diverse functions in the eukaryotic cell ranging from roles in cell motility, cell division, vesicle trafficking to functions in cell signalling and regulation of transcription (Hofmann, 2009; Pollard and Cooper, 2009). A critical property of actin is its ability to form filamentous polymers (F-actin), and a plethora of proteins are involved in the highly dynamic regulation of F-actin formation (Lee and Dominguez, 2010). Actins are highly conserved proteins that often exist in multiple isoforms in the eukaryotic cell and their expression is regulated both spatially and temporally during development (Wagner et al., 2002; Tondeleir et al., 2009; Perrin and Ervasti, 2010). The number of conventional actin genes varies among eukaryotic organisms. A few single cell eukaryotes, such as Saccharomyces cerevisiae, Toxoplasma gondii, and Trypanosoma brucei encode a single actin gene, which results in lethality when targeted with gene ablation approaches (Shortle et al., 1982; Dobrowolski and Sibley, 1996; Garcia-Salcedo et al., 2004). Many organisms, however, have several conventional actin genes, and a fundamental question in understanding the function of actin is whether different isoforms serve specific functions in the cell.

Apicomplexan parasites all encode one major actin isoform, here termed actin I. Because of a vital role in asexual parasites, e.g. Plasmodium blood stages and T. gondii tachyzoites (Dobrowolski and Sibley, 1996), actin I has not been studied by molecular genetics approaches yet. Studies with actin inhibitors assigned central functions to actin as part of the machinery responsible for the parasite locomotion and host cell invasion (Miller et al., 1979; Dobrowolski and Sibley, 1997). In addition, actin I may have a role in endocytic uptake of haemoglobin during intraerythrocytic development of P. falciparum (Smythe et al., 2008). All apicomplexan parasites also contain a number of actin-related and actin-like proteins (Gordon and Sibley, 2005; Gordon et al., 2010; Siden-Kiamos et al., 2010). Remarkably, Plasmodium species stand out in that they all encode a second conventional actin, termed actin II. Early studies reported expression of actin II at the gametocyte stage of the Plasmodium life cycle (Wesseling et al., 1989) although the function of the gene product has not been investigated.

In this study, we analysed the expression and function of P. berghei actin II. The results reveal that this gene has central and pleiotropic roles in the development of the male gamete of the malaria parasite.

**Results**

P. berghei actin II: comparison to actin isoforms of other organisms

Orthologous genes encoding actin II have been identified in several Plasmodium species (for gene identifiers see Fig. S1); the overall identity between the actin II proteins of the different species ranges between 92% and 99%. The gene structure is also highly conserved, with the open reading frame (ORF) encoded by two exons interrupted by an intron at 446 nucleotides downstream of the initiation ATG (Fig. S3A).

A comparison of the P. berghei actin II protein (PBANKA_103010) with the major actin I (PBANKA_145930), reveals that the two proteins are more distantly related (78% identity, Fig. 1A). Both actins deviate from the well-studied actin orthologues from other organisms, here exemplified by Drosophila melanogaster Actin-5C, in approximately the same regions in the sequence; actin II being the more divergent of the two. The majority of the side-chains that are involved in actin–actin contacts within the filament (Oda et al., 2009; Fujii et al., 2010; Murakami et al., 2010) are strictly conserved in both Plasmodium actins. Only one divergent residue within the actin–actin contacts has a substitution in P. berghei actin II that may potentially affect the function; however, this will have to be explored experimentally. A good fidelity structural model of P. berghei actin II could readily be built (Fig. S1A); of all actin crystal structures available in the Protein Data Bank (PDB) the structure of D. melanogaster Actin-5C (Nair et al., 2008) proved to be the best template. The model suggests that actin II is a conventional actin with the potential to form microfilaments.

The phylogenetic relationships between the two Plasmodium actins (actin I and actin II) were investigated through Bayesian Inference and Neighbor-Joining phylogenetic analyses. These approaches aimed to address the crucial questions if a related gene could be detected in other Alveolata and whether actin I and actin II genes split before or after the divergence of the diverse species of the genus Plasmodium. We compared the nucleic acid (Figs 1B and S1B) and the protein (Fig. S1C) sequences of the two Plasmodium actin-encoding genes with related genes from other members of the Alveolata superphylum (Apicomplexa, Dinoflagellata, Ciliophora).

In concordance with previous analyses (Wesseling et al., 1988; Gordon and Sibley, 2005) all Plasmodium sequences were clustered together with high statistical support [1.00 posterior probability (pp) and 97% bootstrap support (bs)]. Within this clade, the actin I sequences consist of a subclade (1.00 pp and 99% bs), which has a sister group relationship with the subclade of actin II sequences (1.00 pp and 100% bs) (Fig. 1B). Considering the correctness of the Plasmodium species recognition, our results support the notion that the two actins (I and II) evolved in the ancestral form of the Plasmodium species, which then diversified in multiple lineages that are recognized today as distinct species.
In blood stages actin II is only expressed in gametocytes

In order to investigate actin II promoter activity, we generated and imaged a transgenic reporter parasite line (Fig. 2), which comprised 1.2 kb of the actin II 5′ flanking region (5′-FR) fused to GFP. A GFP signal could be detected in both male (Fig. 2A and C) and female activated gametes (Fig. 2B). After normalization for autofluorescence of non-GFP-expressing WT parasites, the fluorescence intensity of the males was about ninefold stronger than that of females (Fig. 2D), while no signal was detected in asexual stages.

The absence of promoter activity in the asexual stages contradicts published data, where expression was described for asexual blood stages as well (Hall et al., 2005). To resolve this apparent discrepancy, we further analysed the expression of the gene by RT-PCR analysis. Actin II transcripts were profiled in WT parasites (ANKA 2.34) and two strains that are reported not to produce gametocytes (Fig. 2E).

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to develop gametocytes (ANKA 2.33 and HPE). For comparison we included two transcripts, which have been reported to be gametocyte-specific (Pace et al., 2006; Ponzi et al., 2009) (Fig. 2E). The three tested transcripts were detectable in the ANKA 2.33 strain, but not in HPE (Fig. 2E), indicating that the 2.33 line is not fully deficient in gametocyte production. We detected cells, which were reminiscent of gametocytes, although smaller, in Giemsa-stained smears of 2.33; these were not found in the 2.34 or HPE strains. We therefore concluded that early stages of gametocytes are present but blocked in their further development in the 2.33 strain. This finding provided the opportunity to determine the temporal profile of actin II expression (Fig. 2E), as an infection of cultured blood stages of this strain will only contain viable merozoites, from which gametocytes will develop de novo. This is in contrast to the WT where older gametocytes will also be present. After a synchronous infection of ANKA 2.33 cultured blood stages actin II and SET were expressed at an early time point (15 h after infection) and transcription ceased towards later time points, most likely as a result of the degeneration of the gametocytes. Taken together, the results of the GFP reporter line and the RT-PCR analysis provide strong evidence that the gene is specifically transcribed in gametocytes, particularly in male forms, while the promoter is silent in asexual parasite stages.

Localization of actin II in gametocytes

Next we investigated the intracellular localization of actin II. Attempts to develop specific antibodies directed against two separate peptides in the actin II protein were unsuccessful. As an alternative approach, we generated transgenic parasites that express a GFP-tagged copy of actin II; expression was regulated by the same 1.2 kb promoter is silent in asexual parasite stages containing the 5′ and 3′ regions of the gene as targeting sequences flanking the T. gondii dihydrofolate reductase/thymidylate synthetase (DHFR/TS) cassette conferring pyrimethamine resistance (Fig. S3A). Resulting transfectants were genotyped by PCR (Fig. S3B) and Southern blot analysis (Fig. S3D), which confirmed the correct integration of the DHFR/TS cassette. Furthermore, no mRNA corresponding to actin II was produced in the mutant parasite (Fig. 2E, lane 2). Four clones (from two independent transfections) were further analysed. Apparently, asexual blood stages were not affected by disruption of the gene. Successful generation of actII(-) parasites already indicated that the gene is dispensable for asexual parasite growth. The absence of a detectable phenotype is also in good agreement with the lack of actin II expression in the non-gametocyte producing HPE parasites (Fig. 2E, lane 3).

Actin II is necessary for male exflagellation

We analysed the phenotype of the disrupted actII(-) mutant during later stages of the parasite life cycle. Giemsa staining was used to identify male gametocytes. The results of nine experiments showed that the percentage of male gametocytes in the mutant lines (1.9, range 1.0–3.8) was comparable with that of the WT control (2.8, range 1.0–5.7), thus confirming that male gametocytes

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formed in the mutant strains. However, when blood samples containing gametocytes were activated actII(-) parasites were found to be blocked in exflagellation (Fig. 4A), even after prolonged observation (30 min instead of 10). Very rare events were however detected (2 events in 16 experiments, compared with a total of 1504 in the WT).

Next, blood from infected mice was used to seed ookinetecultures. Very few ookinetes were detected in either of the two mutant lines; ookinete conversion was reduced ~50-fold compared with wild type (WT) parasites (Fig. 4B). To determine whether the actII(-) mutants could be transmitted through mosquitoes, we fed Anopheles gambiae mosquitoes on mice infected with WT or mutant parasites. In three separate experiments, not a single oocyst was detected in the mosquitoes fed on actII(-) parasite infected mice, while WT parasites typically resulted in high prevalence of sporozoite-containing oocysts (Fig. 4C).

**Exflagellation is not affected by actin inhibitors**

To investigate whether our molecular genetics data could be supported by pharmacological studies, we determined the effect on exflagellation in WT parasite of three well-characterized actin inhibitors, cytochalasin D, latrunculin B, and jasplakinolide, each with a discrete mode of action (Bubb et al., 1994; Ayscough et al., 1997; Nair et al., 2008). These inhibitors have been previously shown to inhibit gliding motility of Plasmodium ookinetes (Siden-Kiamos et al., 2006). Our results...
revealed no statistically significant effect of any of the three drugs during this short time span of 10 min (Fig. S4), suggesting that either (i) the process of exflagellation is too fast for the drugs to reach their target, (ii) the drugs do not enter the intracellular gametocytes, (iii) actin II does not perform its critical roles in male exflagellation in a filamentous form or (iv) the drugs do not bind to actin II. We consider the fourth alternative less likely, as superposition of the structural model of actin II with crystal structures of actins in complex with cytochalasin and latrunculin (Fig. S1A) revealed only minor differences in the drug binding sites.

Disruption of actin II abolishes male egress from the RBC and axoneme function

Maturation of male gametocytes encompasses egress from the host cell, DNA replication and axoneme forma-
As the incomplete male gametogenesis detected could be the result of a defect in any of these processes, we investigated each of these in the mutant. To determine whether axonemes were formed, we stained activated gametocytes with the TAT antibody directed against tubulin and the anti-SET antibody to distinguish the nuclei of male gametocytes (Fig. 5A). The SET staining was comparable between the mutant and parental strain. The TAT antibody revealed fully formed axonemes in both strains. However, in WT parasites some axonemes were extending out from the residual cell and nucleus, while in actll(-) parasites they were tightly wrapped around the SET-stained nucleus (Fig. 5A). This, together with the live observations of exflagellation described above, indicated that axonemes were assembled but formation of active, beating flagella failed.

A detailed TEM analysis of the mutant male gametocytes (Fig. 5B and C) was performed in parallel with the previously described ΔPbmap-2 parasites (Tewari et al., 2005) (Fig. 5D). This mutant was reported to egress from the host cell and to undergo normal DNA replication and axoneme formation. However, the latter were never activated and, thus, exflagellation did not take place. Our TEM analysis was fully consistent with these findings. In the actll(-) mutant samples, we could also detect fully formed axonemes (arrows and asterisks in Fig. 5B and C). In both mutants the axonemes were located in the cytoplasm of the male gametocyte. This is in contrast to WT parasites where the axonemes rapidly exit from the gametocyte cytoplasm (Sinden et al., 1976; Ponzi et al., 2009). Also, the secretory organelles called osmiophilic bodies (Hayton and Templeton, 2008) were detected in the actll(-) mutant (Fig. 5B, inset), as well as in the

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\(\Delta Pbmap-2\) strain. An important difference between the two strains was that in the actII(-) mutant the gametocytes had not egressed from the host cell, as revealed by intact PV and RBC membranes, while in the \(\Delta Pbmap-2\) both membranes were disrupted indicative of successful egress.

The nuclei of the actII(-) mutant were filling a large area of the cell, suggesting that the genome had replicated to eight copies. This was confirmed by measuring the DNA content from microscopy images of activated male gametocytes (Fig. 5E). Also, the typical budded appearance was often observed and, moreover, in some sections areas of condensed DNA could be detected.

Taken together, these findings show that the actII(-) parasites are deficient both in egress from the host cell and in the formation of beating flagella, while axonemes are assembled and DNA replication proceeds normally.

Female gametocytes lacking actin II are fertile

Our TEM analysis indicated that the actII(-) mutation had no effect on female gametocytes as the females had escaped the PV and RBC membranes (Fig. 6A). In addition, we could detect females in typical mating centres (Fig. 6B). As stated above (Fig. 4B), the females were also Pbs21-positive, a widely used marker for maturation to female gametes. To rule out any deficiencies in the fertility of the females we crossed the mutant strain with two lines, \(\Delta p47\) forming only fertile males and \(\Delta p48/45\) producing only fertile females. The experiment was repeated three times; SD is indicated in darker gray.

The actII(-) mutant can be partly rescued by gene complementation

We next attempted to restore the function of the mutant by complementing the actII(-) strain with the WT actin II gene under control of its own promoter (Fig. S5A and B). In this complemented parasite line, termed actIIIC, actin II transcripts were readily detectable by RT-PCR (Fig. 2E, lane 9). The complemented parasites were
tested for exflagellation and ookinete conversion in parallel with WT parasites and the actII(-) mutant (Fig. 4D and E), which revealed partial complementation. Exflagellation events were readily detected but, compared with WT parasites, the number of events was reduced. Ookinetes were also formed; however, ookinete conversion reached only about half the value of the WT. In this series of experiments, neither exflagellation events nor ookinetes were detected in the knockout mutant.

**Actin I re-localization in activated male gametocytes is actin II-dependent**

Next, we investigated whether the localization of the major actin I was affected in the actII(-) mutants (Fig. 7). Non-activated WT gametocytes (Fig. 7: a–c) displayed a diffuse actin I staining of both nucleus and cytoplasm as described above. The same pattern was seen in actII(-) mutant male gametocytes (Fig. 7: g–i) and in the complemented parasites (Fig. 7: m–o). However, in activated gametocytes there was a dramatic difference (compare Fig. 7: d–f, j–l and p–r). Whereas actin I in WT parasites was redistributed and found exclusively in the cytoplasm, in the actII(-) mutants actin I remained in the dual localization, i.e. intra-nuclear and diffuse cytoplasmic. This deficiency was fully restored in complemented actIIC gametocytes. Together, comparative imaging of actin I in WT, actII(-) and actIIC parasites suggested that the actin I redistribution upon activation of male gametes requires the presence of actin II.
Discussion

This report presents evidence for a crucial role of the *Plasmodium* actin II isoform in the development of the male gamete. Disruption of the gene had no effect on the asexual parasite stages of the vertebrate host and mutant females were fertile and displayed normal morphology. In contrast, there was a dramatic impairment in egress, the essential step in the maturation of the male gamete, and a concomitant 50-fold decrease in ookinete formation, which resulted in a complete block of mosquito transmission. Analysis of a GFP-tagged version of the actin II protein revealed that it was localized in the cytoplasm of both male gametocytes and activated gametes. We could not detect the protein in the male gamete, although a recent report suggested that actin II is indeed a component of the flagellated gamete (Rupp et al., 2010). Absence of the *P. berghei* actin II fusion protein in gametes may be due to low expression levels as males progress towards maturation. While we cannot exclude the possibility that *P. falciparum* and *P. berghei* may substantially differ in actin II regulation, an actin II-specific antibody will be necessary to resolve this apparent discrepancy. Recognition of multiple bands in Western blot analysis of the actin II protein revealed that it was localized in the cytoplasm of both mature male gametocytes and activated gametes. This re-localization of actin I to the cytoplasm was apparently dependent upon the presence of actin II as it did not take place in the actII(-) mutant.

Previous work [reviewed in (Sinden et al., 2010)] has revealed the importance of kinase signalling during exflagellation, and several kinases have been shown by reverse-genetic approaches to be essential for male gamete development. Of special interest here is the mutant phenotype of ΔPbmap-2 (Khan et al., 2005; Rangarajan et al., 2005; Tewari et al., 2005). Comparing the actII(-) and the ΔPbmap-2 mutants reveal some interesting similarities. In both cases, DNA was replicated normally, and axonemes were assembled, although they were not beating and remained in the parasite cytoplasm. An important difference is that actII(-) parasites are blocked in egress from the surrounding host cell, while in ΔPbmap-2 parasites the PV and RBC membranes are ruptured. The phenotype of the ΔPbmap-2 parasites shows that flagellar beating is not a prerequisite for egress. Another mutant, lacking the CDPK4 kinase, was also found to rupture the surrounding membranes although in this case axoneme assembly was completely blocked (Bilker et al., 2004).

MDV-1/PEG3 is a protein of the osmiophilic bodies and has been shown to function in male gametocyte egress, although it is also required in the females (Lal et al., 2009; Ponzi et al., 2009). TEM analysis revealed that both PV and RBC membranes were intact in the Δmdv-1/peg3 mutants, and the flagella were seen beating trapped inside the host cell (Ponzi et al., 2009). Osmiophilic bodies were observed and the MDV-1/PEG3 protein was present in the actII(-) mutant (I. Siden-Kiamos, unpublished). The lack of reagents specific for these organelles did not allow us to investigate this further and it therefore remains an open question whether actin II may have a role in the function of the osmiophilic bodies.

Actin and actin dynamics have been implicated in assembly and function of axonemes in such different cell types as *T. brucei* (Garcia-Salcedo et al., 2004), *Leishmania donovani* (Tammana et al., 2008), and epithelial cells from vertebrates (Molla-Herman et al., 2010; Ravanelli and Klingensmith, 2011). Although still not understood in detail, actin in these cells probably has a role in vesicular trafficking and is not a direct component of the axoneme. Great care should be exerted when attempting to compare these systems with the *Plasmodium* male gamete formation as the underlying processes show striking differences, but a similar role of actin II can not be excluded. On the other hand, actin has been genetically identified as an important component of the inner dynein arm in *Chlamydomonas reinhardtii* axonemes (Kato et al., 1993; Kato-Minoura et al., 1997). It remains a possibility that the paralysed axonemes in the actII(-) parasites are a direct result of actin II missing from the organelle. We note that an interaction of the dynein light chain 1 (DLC1) with actin I in asexual blood stages of *P. falciparum* was described recently (Daher et al., 2010), and this may be an interesting issue to explore further for actin II.
The pleiotropic phenotype of the actII(-) mutant, in combination with the lack of refined tools, such as specific antibodies and a conditional system for gene regulation, to study this stage of the parasite, makes it currently difficult to pinpoint the exact molecular aspects in which actin II is involved. Nevertheless, the identification of the essential role of actin II in the obligate sexual stage, opens possibilities for further studies, which in the future may suggest targets for transmission-blocking approaches.

Taken together, our results show that actin II has an essential role in male gametocyte development, functioning in several discreet processes. We speculate that utility of two separate actin isoforms with different kinetic properties could be an ancestral aspect of the regulation of actin functions in the absence of a large repertoire of actin regulatory proteins, as seen in Plasmodium (Baum et al., 2006; Sattler et al., 2011). Interestingly, the vital and pleiotropic functions for actin II in male gametogenesis coincide with host switching from warm-blooded Mammalia to the insect vector. The specific, essential role for maturation of male gametes inside the Anopheles midgut therefore provides a plausible explanation for the restriction of actin II to arthropod-transmitted malarial parasites.

**Experimental procedures**

**Phylogenetic analysis**

Actin DNA sequences of 32 Alveolates were aligned in ClustalX (Thompson et al., 1997) and Bayesian Inference (Huelsenbeck and Ronquist, 2001) and Neighbor-Joining (Saitou and Nei, 1987) phylogenetic analyses were conducted in MrBayes (v.3.1.2) (Ronquist and Huelsenbeck, 2003) and PAUP* (v.4.0b10) (Swofford, 2002) respectively. For MrBayes analysis, a fine partitioning strategy was assessed: 1st, 2nd and 3rd positions separately. Each partition was assigned best-fit substitution models using the Akaike information criterion (AIC) (Akaike, 1974) implemented in MrModeltest v2.2 (Nylander et al., 2004) and PAUP* Bayesian Inference analysis was conducted under the General Time Reversible (GTR) (Rodriguez et al., 1990) model of evolution [with a proportion of invariable sites (I) and shape (G) for the first and the third codon positions and GTR + G for the second codon position]. Four runs and eight chains (comprising one cold chain and seven incrementally heated chains and each chain was started from random trees) for each run were performed for 107 generations and the current tree was saved to file every 100 generations. This generated an output of 105 trees. The −lnL stabilized after approximately 106 generations and the first 105 trees (10% ‘burn-in’ in Bayesian terms) were discarded as a conservative measure to avoid the possibility of including random, suboptimal trees. A majority rule consensus tree (‘Bayesian’ tree) was then calculated from the posterior distribution of trees. For the NJ analysis, confidence in the nodes of NJ tree was assessed by 1000 bootstrap replicates (Felsenstein, 1985).

Moreover, in order to examine the effects of mutations in the genomic G + C content of species, a protein Bayesian phylogenetic analysis using the Jones-Taylor-Thornton (JTT) amino acid replacement model (Jones et al., 1992) was performed (for details see Supporting information).

**Parasite strains**

The following *P. berghei* strains were used: ANKA 2.34, a gametocyte producing strain referred to as WT, ANKA 2.33, a derivative reported to be devoid of gametocyte development, and HPE, a second non-gametocyte producing strain (Janse et al., 1989; Dearsly et al., 1990). The Δ47 (Khan et al., 2005), Δ45/48 (van Dijk et al., 2001) and ΔPbm-2 (Tewari et al., 2005) mutants have been described. Parasites were maintained in Theiler's Original mice. All animal work was carried out in full conformity with Greek and German regulations, and the protocols were approved by the Ethics Committee of FORTH and the Berlin State Authorities (LAGeSo Reg# G0469/09) respectively.

**Parasitology methods**

The ANKA 2.33 strain was used to determine the temporal gene expression profile from synchronized parasites. Mixed blood stages were obtained from infected blood with a parasitaemia 3–5% and schizonts were cultured overnight at 37°C, purified on a 15% Nycodenz gradient and then injected intravenously in recipient mice (Janse and Waters, 1995). mRNA was prepared from samples isolated at different time points after infection. Samples from a synchronous WT infection contain a mixture of different stages of gametocytes, as they survive the in vitro culture preceding the infection. In the 2.33 strain no gametocytes will be transferred with the schizonts, and all gametocytes will therefore develop synchronously from the invading merozoites.

Exflagellation was induced by incubating gametocytes at 19°C with exflagellation medium (complete ookinete culture medium supplemented with 50 μM xanthurenic acid). Ookinete cultures were cultured in vitro as described (Rodriguez et al., 2002). For cross-fertilization experiments ookinete cultures were seeded with equal volumes of blood from mice infected with ANKA 2.34, Δ47, Δ48/45 or actII(-). To determine the oocyte conversion rate (the percentage of female gametes developing into oocytes), samples from an in vitro oocyte culture were incubated with the mAb 13.1, recognizing the Pbs21 surface antigen present on female gametes, zygotes and ookinetes (Winger et al., 1998). Mosquito infections were done in A. gambiae mosquitoes strain G3 and dissections for oocyst counts were done at 12 days after feeding. The strain expressing the GFP-actin II fusion protein from an episomal construct was grown in animals, which were continuously treated with pyrimethamine (14 μg ml⁻¹) in the drinking water as described (Janse et al., 2006) to avoid the loss of the episome during schizogony.

**DNA constructs**

The primers used for the constructs are summarized in the Table S1. Plasmids and sequences of pL0001, pL0004 and pL0008 are available from MR4 (http://www.malaria.mr4.org).

actII(−). The gene knockout construct was made in the standard vector pL0001. 473 bp of the 5’ region of actin II (including 69 bp of the 5’-FR) and a 494 bp fragment of the 3’ end of the actin II
ORF were amplified from *P. berghei* genomic DNA (gDNA). The fragments were cloned into the KpnI and HindIII sites and the EcoRI and BamHI sites respectively. The plasmid was digested with KpnI and BamHI before transfection.

**actII complementation construct.** The 3′-UTR of the *P. berghei* DHFR-TS gene was amplified from gDNA and inserted into Blue-script KS using the EcoRI and KpnI sites. This plasmid was named pDHFR/BS and fragments were inserted into this plasmid using the SmaI and EcoRI sites. A single fragment corresponding to 1226 bp of the actin II 5′-FR and 1264 bp of the act II ORF was amplified by PCR from *P. berghei* gDNA. The fragment was inserted in pDHFR/BS. Finally, the cassette was introduced into the pL0008 vector using the unique KpnI site. Before transfection the plasmid DNA was linearized with Apal.

**actII promoter reporter construct.** To analyse the promoter activity of the actin II gene, 1.2 kb of the 5′-FR was amplified from gDNA using primers 5PbACT2-F and 5PbACT2-R and cloned into a standard transfection vector containing the GFP coding region with the non-regulatory *P. berghei* dihydrofolate synthase/folypolyglutamate synthase 3′UTR (PBANKA_134000). In addition, this vector contains the pyrimethamine-resistant DHFR/TS cassette and two sequences for targeted homologous recombination by double crossover in an intergenic locus on *P. berghei* chromosome 12 (between PBANKA_122210 and PBANKA_122220; TWAK, unpublished). The resulting plasmid, p5PbACT2-GFP, was linearized with PvuI simultaneously cutting *P. berghei* dihydrofolate synthase and 5′FRSH and 5′FR2USR. GFP was amplified with CFPA2P1 and CFPA2P2 and the 1264 bp fragment of the actin II ORF with CFPA2P3 and RevActII. The resulting fused gene had a linker encoding the amino acids Ala,ValAspAla, inserted between GFP and actin II. The amplified fragments were inserted in pDHFR/BS as described for the complementation construct and finally the complete cassette was inserted into the pL0004 transfection vector. The vector was introduced as an episome. Exflagellation and ookinete formation of the resulting strain were compatible with the WT.

**Transfection, cloning and genotyping**

Parasites were transfected and cloned as described (Janse et al., 2006). Correct integration was verified using PCR with the primers indicated in the Table S1; primers L635, L665, L739 and L740 were described previously (Franke-Fayard et al., 2004). Southern blot analysis of the actII(-) parasites was performed as described (Siden Kiamos, 1997). In brief, gDNA was digested with Spel and separated on an agarose gel. The DNA was then transferred to a nitrocellulose membrane filter and hybridized at 60°C with mixed probes corresponding to the 5′ and 3′ targeting regions of actin II.

**RT-PCR**

Total RNA was isolated from 100 µl of infected blood using the TRI reagent from Sigma. The cells were pelleted and then resuspended in 500 µl TRI, 100 µl of chloroform was added and centrifuged at 4°C at 15 000 g. RNA was recovered, washed in 70% ethanol and resuspended in diethyl dicarbonate treated water. cDNA was synthesized using the Thermoscript RT-PCR system Kit (Invitrogen) according to the protocol and the primers used for PCR using Taq polymerase (Promega) are shown in Table S1.

**Fluorescence microscopy of 5PbACT2-GFP parasites**

Five microlitres of blood from an infected mouse was mixed with 395 µl of exflagellation medium and distributed onto poly-L-lysine coated cover slips. After 14 min at room temperature (RT), the cover slips were transferred to a 24-well plate containing 500 µl of 4% EM-grade paraformaldehyde and 0.0075% EM-grade glutaraldehyde in microtubule stabilizing buffer (MTSB, 10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM MgCl2 pH 6.9), fixed for 30 min and washed with PBS. Cells were permeabilized for 10 min with 0.5% Triton X-100 in PBS and blocked 1 h with 5% foetal calf serum (FCS) in PBS. The samples were incubated with the anti-SET antibody in 5% foetal calf serum in PBS overnight at 4°C and with Alexa Fluor 546 conjugated anti-rabbit antibody (Invitrogen). Nuclei were visualized with DRAQ5 (Axxora). Cover slips were mounted with Fluoromount-G (Southern Biotech). Images were recorded on a Leica TCS SP-1 confocal microscope.

**Immunofluorescence analysis**

Gametocyte containing blood was activated in exflagellation medium at 19°C before fixation for 1 h in 4% paraformaldehyde in MTSB or immediately added to the fixative. All steps were carried out at RT, unless indicated otherwise. The parasites were added to poly-L-lysine coated cover slips placed in 24-well plates, centrifuged at 500 g for 10 min, and the fixative removed. Samples were permeabilized with 0.5% Triton X-100 in PBS for 10 min, rinsed twice in PBS and the primary antibodies were added diluted in PBS with 5% normal goat serum. Incubation was either 1 h at RT or overnight at 4°C. Cells were washed twice with PBS for 5 min and incubated with secondary antibodies, washed twice with PBS for 5 min before mounting in Vectashield (Vector laboratories). The samples were analysed using Bio-Rad confocal microscope attached to a Zeiss Axioskop 2 plus microscope. Images were analysed with ImageJ software (http://rsbweb.nih.gov/ij/).

**Antibodies**

The anti-SET antibody has been described (Pace et al., 2006). The mAb TAT was raised against *T. brucei* tubulin, but also recognizes *P. berghei* tubulin (Billker et al., 2002). Actin I was detected using mAb 224–236-1 (Westphal et al., 1997), used previously to visualize actin I in *P. berghei* ookinetes (Siden-Kiamos et al., 2006). The anti-GFP antibody was purchased from Invitrogen. Secondary antibodies were anti-mouse (Alexa-488, Alexa-555) and anti-rabbit (Alexa-488, Alexa-546) conjugated to Alexa Fluor (Invitrogen) or anti-rabbit conjugated to Cy-3 (Jackson Research).

**Determination of DNA content**

Samples from infected mice were incubated for 8 min at 19°C in exflagellation medium, fixed in 2% formaldehyde in PBS for
Transmission electron microscopy

Infected mice were treated for 36 h with 15 mg ml⁻¹ sulfadiazine in the drinking water (Beetsma et al., 1998). The following stages were captured at RT. The infected blood was immediately diluted 20-fold in gametocyte medium (GM) (RPMI 1640 containing 25 mM HEPES, 2 mM L-glutamine, supplemented with freshly made 0.2% NaHCO₃ and 1 mg ml⁻¹ BSA, pH 7.3). The gametocytes were layered on a cushion of 15% Nycodenz diluted in GM and centrifuged at 500 g for 20 min. The interphase was recovered and the gametocytes were pelleted at 500 g for 5 min and resuspended in GM. Gametogenesis was induced by diluting the gametocytes 1:5 in exflagellation medium and incubated at 19°C. After 15 min the gametocytes were fixed in 2% glutaraldehyde, 2% paraformaldehyde, 2 mM CaCl₂ in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4°C, and processed according to Perry and Gilbert (Perry and Gilbert, 1979). Paraformaldehyde, OsO₄ in 0.1 M sodium cacodylate buffer for 1 h at RT, treated with 1% tannic acid in 0.05 M cacodylate buffer for 30 min and rinsed in 1% sodium sulphate in 0.05 M cacodylate buffer for 10 min. Postfixed specimens were washed, dehydrated through a graded series of ethanol solutions (30–100% ethanol) and embedded in Agar 100 (Agar Scientific Ltd, UK). Ultrathin sections, obtained by an UC6 ultramicrotome (Leica), were stained with uranyl acetate and lead citrate and examined by an EM208 Philips electron microscope.

Acknowledgements

We thank G. Vrentzos and L. Spanos for technical support and Maria Kydonaki and Andreas Kunze for help in the initial stages of the analysis. We are grateful to Oliver Billker, Robert E. Sinden, Dominique Soldati and Marta Ponzi for gifts of antibodies. R.M. was supported by Intermal, a Marie Curie Initial Training Fellowship, and Canada (http://www.thesgc.org/about/sponsors.php/). H.S. is supported by The Structural Genomics Consortium, supported by a long-term fellowship of the European Molecular Biology Organization (ALTF-763–2006) and a Max Planck fellowship. H.S. is supported by The Structural Genomics Consortium, a registered charity (1097737) receiving funds from Sweden, UK and Canada (http://www.thescg.org/about/sponsors.php/).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. A structural model of P. berghei actin II and a comparative phylogenetic analysis.

A. Model of the structure of P. berghei actin II, based on the crystal structure of D. melanogaster Actin-5C (PDB entry 3eks) with N- and C- termini indicated. Also shown are the co-ordinates for tightly bound Ca2+ (green) and ATP (sticks). The structures of cytochalasin (pink carbon atoms; taken from PDB entry 3eks) and latrunculin (green carbon atoms; taken from PDB entry 2q0u) were superposed onto the model of actin II. The details of the interaction of actin with jasplakinolide are unknown. The model was constructed using Swiss-Model (Arnold et al., 2006). The geometry of the model was checked with MolProbity (Davis et al., 2007) and found to be acceptable (0.27% Ramachandran outliers; 2% poor rotamers; 1 Cβ bond deviation). The final figure was generated with ESPript (Gouet et al., 1999).

B. Comparative phylogenetic analysis of the set of actin isoforms from Fig. 1. Shown are nucleic acid sequence-based (B) and amino acid sequence-based (C) phylogenetic analyses. Posterior probabilities are indicated at each node. Black arrows indicate posterior probabilities are indicated at each node. Black arrows indicate
cate clades with no significant support under the posterior probabilities. The amino acid tree has the similar, although unresolved, topology as the nucleic acid tree. The main subclades of the nucleic acid tree (i.e., all the Plasmodium actin I sequences, all the Plasmodium actin II sequences) exist, but the phylogenetic relationships among them should be considered as unresolved (not significant posterior probabilities, < 0.95). This is probably due to the fact that the nucleic acid analysis is more informative than the corresponding amino acid analysis, due to the threefold number of characters. Light shade: Apicomplexa, dark shade: Plasmodium spp. The number of characters. Light shade: Apicomplexa, dark shade: Plasmodium spp.

Fig. S3. Generation of P. berghei actin II knockout mutant and genotyping.
A. Top: Maps of the WT actin II locus, the disruption vector p.actII, and the actin II locus after integration of p.actII. The two fragments for targeting the insertion in the gDNA by double cross-over are shown as double arrows in blue. Primers used for diagnostic PCR (black arrows), the expected fragments (purple double arrows) and their size are indicated. Middle: The disruption vector p.actII. Note that the DHFR/TS cassette is not shown to scale. Bottom: Locus after integration of the DHFR/TS cassette (not to scale).

B. Diagnostic PCR reactions on gDNA from WT and mutant clones. Left panel: WT (lanes 1, 4 and 7) and the clones actII(-) cl1-2 (lanes 2, 5 and 8) and actII(-) cl1-3 (lanes 3, 6 and 9), originating from first transfection. Right panel: Mutant clones actII(-) cl2-1 (lanes 1, 4 and 7), actII(-) cl2-5 (lanes 2, 5 and 8), WT (lanes 3, 6 and 9). The primer pairs are indicated on the top.

C. Southern blot analysis of the actII(-) mutant. (C) The actin II locus of WT and knockout parasite after integration of the DHFR/TS cassette. The sites of the restriction enzyme Spel and the sizes of the fragments generated by digest are indicated. (D) Southern blot analysis of gDNA originating from WT and the two actII(-) clones digested by Spel. The blot was hybridized with the two fragments used to construct the targeting plasmid (red lines in C).

Fig. S4. Actin inhibitors do not block exflagellation. P. berghei-infected blood was incubated under exflagellation conditions in medium containing 5 μM latrunculin B, 5 μM cytochalasin D or 250 nM jasplakinolide. Exflagellation events were scored under the microscope 10 min after addition of the infected blood. The treated samples were normalized to untreated control samples. (n = 3, the SEM is indicated in darker green). Differences are not significant (Student’s t-test).

Fig. S5. Complementation of the P. berghei actin II knockout mutant.
A. Schematic representation of the strategy for generating a functional copy of the actin II gene in the knockout mutant. The genomic locus of actin II, including 1.2 kbp of the 5′-FR, the two exons and the intron interrupting the ORF, was fused to the 3′UTR derived from the P. berghei dhfr-ts gene and the cassette was inserted in the pLO008 plasmid vector to generate the construct pactII. Note that the diagram of the plasmid backbone is not scaled (dotted line). The construct was linearized with the restriction enzyme Apai and integrated via a single cross over in the c/d ssu-rma locus of the actII(-) mutant. Primers used for the analysis of correct integration as well as the size of the fragments generated are indicated.

B. PCR analysis of gDNA prepared from the actII complementation line (lane 1, 4, 7), WT (lane 2, 5, 8) and actII(-) mutant (lane 3, 6, 9). The primer pairs are indicated above the gel and DNA size markers (in kb) on the left.

Table S1. Primers used for DNA constructs, analysis of correct integration and RT-PCR analysis.

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