A Malaria Parasite Formin Regulates Actin Polymerization and Localizes to the Parasite-Erythrocyte Moving Junction during Invasion

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Summary

Host cell invasion by malaria parasites critically requires the turnover of short actin filaments; however, the factors that regulate filament nucleation and polymerisation are unknown. Malaria and other apicomplexan parasites lack Arp2/3 complex, an actin nucleator widely conserved across eukaryotes, but do express formins. Here, we demonstrate that one of two malaria parasite formins, \textit{Plasmodium falciparum} formin 1, and its orthologue in the related parasite \textit{Toxoplasma gondii}, follows the predicted site of the activated actomyosin motor during cell invasion - the moving tight-junction complex. Furthermore, \textit{in vitro}, the PfFormin1 actin-binding, formin homology 2 (FH2) domain is a potent nucleator, stimulating actin polymerisation and, like other formins, localizing to the barbed end during filament elongation. Formin 1 is the first actin regulator demonstrated to be active during apicomplexan parasite motility and, given the essential role actin plays in cell invasion, highlights formins as important determinants of malaria parasite pathogenicity.
Introduction

The phylum Apicomplexa comprises a group of single celled early branching eukaryotes that includes malaria parasites and other pathogenic protozoa of humans and livestock. Instead of using either cilia or flagella (based on microtubule axonemes) or actin assembly driven by Arp2/3 complex to move, apicomplexan parasites use a unique actomyosin-based, substrate-dependent form of locomotion, called gliding motility, to cross epithelial barriers and invade target host cells (Baum et al., 2006a; Morrissette and Sibley, 2002). The molecular motor, thought to drive this process, is housed in a space (referred to as the supra-alveolar space [Raibaud et al., 2001]) within the parasite pellicle that lies between the outer plasma membrane and the inner membrane complex (Baum et al., 2006a; Morrissette and Sibley, 2002). The motor consists of a single-headed myosin found only in Apicomplexa (Foth et al., 2006; Meissner et al., 2002) that tread along short, dynamic, actin filaments (Sahoo et al., 2006; Schmitz et al., 2005; Schuler et al., 2005). Myosin is anchored in the external face of the inner membrane complex (Gaskins et al., 2004), whilst actin filaments, lying within the supra-alveolar space (Dobrowolski et al., 1997; Patron et al., 2005; Shaw and Tilney, 1999; Wetzel et al., 2003), form a bridge with the extra-cellular environment via the cytoplasmic tail of transmembrane-bound adhesins secreted onto the parasite surface (Baum et al., 2006b; Jewett and Sibley, 2003). The binding of these adhesins to the host surface and their rearward movement, driven by myosin via actin, propels the parasite over the cell substratum or powers active penetration of target cells (reviewed in [Baum et al., 2006a]). It has been known for some time that drugs that disrupt actin polymerisation or filament turnover block motility and cell invasion of apicomplexan
parasites, demonstrating the essential role of dynamic actin (Dobrowolski and Sibley, 1996; Miller et al., 1979; Mizuno et al., 2002; Pinder et al., 1998; Wetzel et al., 2003). However, the cellular factors regulating actin in Apicomplexa and the signal cascades that lead to polymerisation are not known.

Apicomplexa lack genes for many known actin regulators (Baum et al., 2006a), including the subunits of Arp2/3 complex, which nucleates actin filaments in plants, amoebas, fungi and animals (Pollard, 2007). Apicomplexa do have genes for formins (Goode and Eck, 2007; Pollard, 2007), a family of proteins that nucleate unbranched actin filaments for many structures across eukaryotes (Higashida et al., 2004; Martin and Chang, 2006; Michelot et al., 2005; Sagot et al., 2002). The defining feature of formin proteins is the formin homology (FH) 2 domain (Higgs and Peterson, 2005), a region of ~400 amino acids that forms a homodimer (Otomo et al., 2005; Xu et al., 2004), which typically remains bound to the growing barbed end of a nucleated filament (Higashida et al., 2004; Kovar et al., 2006; Romero et al., 2004). Most formins also contain a proline-rich FH1 domain adjacent to the FH2 domain (Higgs and Peterson, 2005), which binds profilin-actin complexes for efficient delivery of new monomers onto growing filaments (Kovar et al., 2006; Kovar et al., 2003; Paul and Pollard, 2007; Romero et al., 2004). A search for proteins containing regions homologous to the FH2 domain of the budding yeast formin Bni1p (residues 1348-1766) (Higgs and Peterson, 2005) in the genome sequence of *Plasmodium falciparum*, the most virulent human malaria parasite, previously identified two very large (>300 kDa) formin-like proteins (Baum et al., 2006a), here called *P. falciparum* formin 1 (PfFormin1) and formin 2 (PfFormin2) (Fig. 1A) [previously called
PfForB and PfForA respectively (Baum et al., 2006a). The genomes of other apicomplexan parasites, including several malaria parasite species, also contain orthologues of 1 and 2-like formins (Baum et al., 2006a). These cluster into two distinct families (Fig. S1), suggesting that each formin may play a conserved role in actin regulation.

Here we show that of the two formins expressed in *P. falciparum*, PfFormin1 localizes precisely with the predicted location of the activated actomyosin motor in invading malaria parasites and is a potent nucleator of actin filaments *in vitro*. Furthermore, we show that the formin 1 orthologue in *Toxoplasma gondii* follows a similar localization during host cell invasion, supporting the notion that motility across apicomplexan parasites is governed by conserved molecular mechanisms. As the first demonstrated actin regulator active during apicomplexan motility, formin 1 represents a potential key effector of host cell invasion and, as such, of parasite pathogenicity.
Results

Differential expression and localization of malaria parasite formins 1 and 2

Microarray data through the lifecycle of the malaria parasite, *P. falciparum*, suggests that the two formins are expressed across asexual and sexual stages of development (Le Roch et al., 2003). To definitively determine their presence in the erythrocytic stages (the lifecycle stage associated with disease) we raised antibodies against recombinant FH2 domains for both *P. falciparum* formins (Fig. 1A). Immunoblots demonstrate that both are expressed during asexual parasite development (Fig. 1B). In agreement with its gene expression profile (Le Roch et al., 2003), maximal expression of PfFormin1 is late in the lifecycle at 40-48 h post invasion (Fig. 1B) a stage when much of the machinery for erythrocyte invasion is assembled. Immunofluorescence microscopy of free merozoites demonstrates that PfFormin1 concentrates at the tip of the parasite (Fig. 1C; white arrow) whilst being absent from earlier trophozoites (Fig. 1C; yellow arrow). In contrast, PfFormin2 expression peaks 24 h post invasion at the mid trophozoite stage (Fig. 1B), a time of parasite growth and nuclear division, and by immunofluorescence localizes diffusely through the trophozoite cell cytoplasm though largely absent from the nucleus (Fig. 1C). Given its pattern of expression and anterior localization we co-localized PfFormin1 with other components known to be important to cell invasion. Immunofluorescence clearly shows that it resides at the apical pole of free merozoites, in front of the bulb of the rhoptry (an apical organelle involved in invasion) (Fig 2A) and anterior to the natural apical break in the inner membrane complex (Fig. 2B; white arrow) (Morrissette and Sibley, 2002). This location coincides with the apical polar rings and the microtubule-organizing centre from which the limited number of merozoite microtubules originate (Morrissette and Sibley,
2002) (Fig. 2C). The region anterior to the apical pole is also the area where actin appears to be concentrated (Fig. 2D) (Mizuno et al., 2002; Shaw and Tilney, 1999; Wetzel et al., 2003). Immunoelectron microscopy of free merozoites using anti-PfFormin1 gives further support to the localization of formin 1 anterior to the rhoptries (Fig. 2F, white arrow heads). Close inspection of the three pellicular membranes again suggests a concentration of labelling at the break in the inner membrane complex, possibly associated with the supra-alveolar space lying between the outer plasma membrane and inner membrane complex (Fig. 2F, inset).

Since profilin is known to play a key co-regulatory role in formin-mediated elongation of actin filaments (Kovar et al., 2006; Kovar et al., 2003; Romero et al., 2004) we raised antibodies against recombinant \textit{P. falciparum} profilin (PfPRF) to investigate its localization and timing of expression. PfPRF appears to be present throughout the asexual cycle, peaking in expression coincidently with PfFormin1 (Fig. 1B), whilst dipping at the time of peak expression of PfFormin2 (Fig. 1B). Localization of PfPRF within free merozoites is diffuse within the parasite cytosol though concentrating towards the parasite apex, where PfFormin1 is most abundant (Fig. 2E). The localization and temporal expression of \textit{P. falciparum} profilin is consistent with it participating in actin polymerisation in spite of the fact that PfFormin1 lacks a canonical poly-proline rich FH1 domain (see below) (Fig. 1A).

\textit{Localization of formin 1 to the merozoite-erythrocyte tight junction}

The molecular interactions between merozoite and erythrocyte that facilitate cell invasion are believed to localize to an electron dense tight junction formed between the opposing
plasma membranes of the parasite and red cell (Miller et al., 1979). Although no components are definitively characterised for this structure in malaria parasites, recent work has identified a rhoptry neck protein, TgRON4, in the related apicomplexan parasite *Toxoplasma gondii* (Alexander et al., 2005), which follows the tight (or moving) junction during host cell invasion (Alexander et al., 2005). We investigated the co-localization of the *P. falciparum* orthologue of RON4 (D. Richard and A.F. Cowman *manuscript in prep*) and PfFormin1 in merozoites arrested using cytochalasin D, following which merozoites form a tight junction but do not invade (Miller et al., 1979). Prior to invasion, PfFormin1 and PfRON4 co-localize at the apical pole of free merozoites (Fig. 3A). However, in the rare event of capturing arrested invasion, fluorescence of both PfFormin1 and PfRON4 appear to split to form two distinct foci (a ring in three dimensions) either side of the merozoite at the interface with the erythrocyte (Fig. 3B). This is consistent with their location at the tight junction during merozoite invasion (Fig 3; schematic).

**The *Toxoplasma gondii* formin 1 orthologue follows the active invasion motor**

Because of the small size (~1.5 μm) and limited viability of merozoites, and given the considerably larger cell size (~7 μm) of *T. gondii* tachyzoites we investigated whether the formin 1 orthologue in this related parasite follows a similar path. Antibodies against *P. falciparum* formins cross react with large proteins (>300 kDa) in soluble extracts from free tachyzoites (Fig. 1B). The specific cross-reactivity of antibodies raised against recombinant PfFormin1 for TgFormin1 was confirmed by immunoblot using extracts from a *T. gondii* line expressing a green fluorescent protein (GFP) tagged TgFormin1 FH2 domain (Fig. S2); the TgFormin1-FH2 domain shares ~50% identity and even greater percentage residue
similarity to the FH2 domain of PfFormin1. Immunofluorescence microscopy of free tachyzoites shows TgFormin1 concentrates at the parasite apical pole, in particular associating with the tubulin-rich conoid of the parasite, which can be seen as an extended structure in free tachyzoites (Fig. 4A; inset yellow arrow) (Morrissette and Sibley, 2002), and radiating back along the cell periphery following, what appears to be, the early portion of the inner membrane complex (Fig. 4A, Movie S1). Furthermore, apical staining can also be seen in the nascent apical complex of daughter cells (Fig. 5C; open arrows), which is similar to that seen for T. gondii dynein light chain and calcium binding proteins recently shown to be associated with the conoid (Hu et al., 2006). It is worth noting that the same study found the formin 1, but not formin 2, orthologue present in conoid-enriched fractions of tachyzoites (Hu et al., 2006). The association of TgFormin1 with the conoid was further investigated in transfectant Toxoplasma parasites expressing yellow fluorescence protein (YFP) tagged \( \alpha \)-tubulin (Striepen et al., 2000) (Fig. 4B). Formin 1 and \( \alpha \)-tubulin clearly co-localize to the conoid in deoxycholate-treated tachyzoites, an extraction process that reveals an intact conoid and microtubules (Hu et al., 2002) (Fig. 4B, yellow arrows). Immunoelectron microscopy of free tachyzoites using anti-PfFormin1 serum suggests a close association of TgFormin1 with apical region and outer-pellicle of the cell (Fig. 4C, white arrows), although there is no close association with a specific cell structure and there is limited (but at a lower percentage) labelling within the central cytosolic region of cells. However, treatment of tachyzoites with Clostridium septicum \( \alpha \)-toxin (Wichroski et al., 2002), which leads to the selective separation of plasma membrane from the inner membrane complex, clearly shows that the formin 1 protein is associated with the
tachyzoite plasma membrane and supra-alveolar space, showing dense staining at the cell periphery (Fig. 4D; closed arrow). This is in contrast to the myosin anchor GAP50, which localizes to the outer face of the inner membrane complex (Gaskins et al., 2004) (Fig. 4D; open arrow).

We investigated the location of formin 1 during tachyzoite cell invasion. In tachyzoites arrested mid-invasion (Kafsack et al., 2004), TgFormin1 progresses down the cell periphery following the moving junction (Fig. 5A-C; white arrows), a structure clearly defined by TgRON4 localization (Alexander et al., 2005) (Fig. 5A). The movement of TgFormin1 with RON4 in tachyzoites has significant parallels with *P. falciparum* merozoites during erythrocyte invasion (Fig. 3B) and strongly supports a role for formin 1 in regulating actin polymerisation during apicomplexan parasite invasion.

*Molecular modelling of the *P. falciparum* formin-actin interaction*

To examine the potential of PfFormin1 and PfFormin2 to nucleate new actin filaments, we undertook molecular modelling of the FH2 domains of both *P. falciparum* formins based on published structures of the FH2 domain of budding yeast formin Bni1p (Otomo et al., 2005; Xu et al., 2004). The FH2 sequences of PfFormin1 and PfFormin2 share ~27% identity with each other and with Bni1p (Otomo et al., 2005; Xu et al., 2004) and share several key residues known to be essential for the interaction of the FH2 dimer with actin (in particular Ile 1431 and a basic residue at 1601) (Otomo et al., 2005) (Fig. S3). Furthermore, models of either formin dimer with *P. falciparum* actin demonstrate that the two primary interfaces with the actin monomer, the knob and post-lasso regions (Otomo et
al., 2005), show conservation in key hydrophobic and polar residues respectively (Fig. 6A-B). Neither *P. falciparum* formin has a clearly defined FH1 domain (Fig. 1A) that would be predicted to recruit profilin (Kovar et al., 2006; Kovar et al., 2003; Pollard, 2007; Romero et al., 2004), the lack of which appears to be common among apicomplexan formins (data not shown). PfFormin2 has two potential PPPP(L/P)P profilin binding sites, while PfFormin1 has only two pairs of proline residues separated by 5 other residues (Fig. 1A).

**Nucleation of actin by malaria parasite formins in vitro**

Given their potential involvement in invasion we investigated whether *P. falciparum* formins nucleate actin filaments *in vitro*. Recombinant proteins comprising truncated and extended PfFormin1 and PfFormin2 FH2 domains were expressed and purified from *E. coli* (Fig. 6C). Extended constructs were designed to incorporate the key regions conserved with Bni1p FH2 and include the most extensive poly-proline stretches towards the N-terminus as potential FH1 domains (Fig. 1A). To address whether these PfFormin1 and PfFormin2 FH2 domains bind to malaria actin we undertook pull-down assays of whole parasite lysate using the recombinant GST fusions. Both extended FH1FH2 constructs successfully precipitated parasite actin, though shorter constructs (lacking the essential alpha-helices A and B required for dimerization, Fig. S3) or GST alone did not, supporting an *in vivo* interaction between parasite formins and actin (Fig. 6D). Given that extraction of high quantity (milligram) and purity actin from malaria parasites is practically impossible (Schmitz et al., 2005), we assayed the nucleation potential of the malaria formins with chicken skeletal muscle actin, which despite its divergence, shares key residues in the necessary formin-actin interfaces that are required for nucleation (Fig. 6A-B and data not
shown). PfFormin1-FH1FH2 and PfFormin2-FH1FH2 both stimulated the rate of assembly of bulk samples of chicken actin monomers (measured by increased fluorescence of pyrenyl-actin) (Fig. 6E-F) demonstrating they are true nucleators. The polymerisation rate depended on the concentration of formin (Fig. 6E-F). Like yeast Bni1pFH1FH2 (Paul and Pollard, 2007), PfFormin1-FH1FH2 strongly stimulates actin assembly at low nanomolar concentrations in the absence of profilin (Fig. 6E), while PfFormin2-FH1FH2 was a less potent stimulator (Fig. 6F). As predicted, the truncated FH2 domains of either *P. falciparum* formin failed to nucleate actin (data not shown). To calculate the concentration of growing ends across formin concentrations in the bulk polymerisation assays, and therefore measure the nucleation activity of each formin (Kovar et al., 2003), we used rates of elongation of single filaments measured with microscopy in the presence of either formin (see below). A plot of ends produced versus formin concentration shows that the nucleation activity of PfFormin1-FH1FH2 is much greater than that of PfFormin2-FH1FH2 (Fig. 6G). The concentration of ends produced by PfFormin1 plateaus at 9.4 nM because elongation of numerous filaments rapidly depletes the pool of actin subunits available for nucleation.

To distinguish the contributions of nucleation and elongation to actin polymerisation stimulated by PfFormin1 and PfFormin2, we used total internal reflection fluorescence microscopy (TIRFM) to measure directly the effect of either recombinant formin on elongation of actin filaments (Kovar et al., 2003) (Fig. 7A-C, supplementary Movies S2-5). Similar to other well-characterized formins (Kovar et al., 2006; Kovar et al., 2003; Paul and Pollard, 2007), we found that FH1FH2 constructs of both PfFormin1 and PfFormin2...
slowed barbed end growth (Fig. 7E-F). Elongation rates in the presence of either formin reduced the average barbed end growth rate from $14.6 \pm 0.8$ subunits/s for actin alone (Fig. 7D) to $9.0 \pm 2.3$ subunits/s for PfFormin1-FH1FH2 (Fig. 7E) and $8.5 \pm 3.4$ subunits/s for PfFormin2-FH1FH2 (Fig. 7F). Filaments growing at rates consistent with actin alone were visible in assays using either formin, suggesting that we observed both free and formin-associated filaments. Moreover, the reduced barbed end elongation rate with formin is not the result of depletion of the actin monomer pool due to a high number of nucleated ends, but rather suggests the formin remains bound to the growing barbed end of a nucleated filament – a phenomenon known as processive association (Kovar et al., 2006; Paul and Pollard, 2007).
Discussion

We have shown that malaria formin PfFormin1 is a potent nucleator of filamentous actin, is present at the apical pole of invading merozoites, and co-migrates during invasion with the merozoite-erythrocyte moving junction – the predicted location of the actomyosin motor that powers parasite motility. Our results suggest that formin 1 is a key effector of actin filament formation required for successful malaria parasite invasion of the erythrocyte, providing the strongest evidence to date for function of an actin regulator in apicomplexan host-cell invasion. Furthermore, this role appears to be essential given the failure of repeated attempts, in different malaria parasite strains, to disrupt the function of the formin 1 gene (data not shown). The implication of PfFormin1 in apicomplexan cell locomotion further expands what is already a diverse range of cellular functions that formin-like proteins play in cytoskeletal assembly and organization (Goode and Eck, 2007; Pollard, 2007).

Preliminary in vitro kinetic data for apicomplexan actin suggest that it should form at very low critical concentrations (Sahoo et al., 2006). However, filamentous actin is strikingly absent from apicomplexan parasite cytosol (Bannister and Mitchell, 1995; Dobrowolski et al., 1997; Field et al., 1993; Shaw and Tilney, 1999). The best estimates for the cellular concentration of monomeric actin in Plasmodium (Field et al., 1993) and Toxoplasma [cited as unpublished data in (Sahoo et al., 2006)] are well above that required for actin to polymerise in vitro, suggesting tightly controlled regulation of actin filament assembly in vivo. The reduced repertoire of known eukaryotic actin regulators (Baum et al., 2006a)
suggests those present play central roles in regulating actin’s state. For example, the actin monomer-binding proteins, namely profilin, actin-depolymerizing factor (ADF)/cofilin and Srv2/cyclase-associated protein (Revenu et al., 2004), are likely to play key roles in preventing spontaneous assembly of filaments. However, whilst these three monomer-binding proteins may be critical in determining availability of actin monomers for polymerisation, it is the localization of factors stimulating polymerisation that will ultimately determine motor engagement. It is the localization and demonstrated biochemical activity of formin 1, as a stimulator of actin polymerisation, that highlight its potential importance to apicomplexan cell motility.

The sub-cellular concentration of formin 1 at the parasite apex and outer pellicle (Fig. 2, 4) and its localization during invasion (Fig. 3, 5) raises several questions about the molecular processes underlying host cell invasion by apicomplexan parasites. During cell invasion, the arrangement of formin 1 behind RON4 at the moving tight junction suggests RON4 is involved with or follows molecular establishment of the tight junction, which then allows recruitment of the activated motor complex. However, it is not clear when actin filaments are first initiated and, as such, when formin 1 is actually active. In repeated immunofluorescence microscopy assays with T. gondii tachyzoites mid-invasion, we consistently observed a proportion of formin 1 fluorescence remaining at the parasite apex past the point of contact with the host cell (Fig. 5A,C). This observation could be interpreted as evidence that formin 1 stimulates actin polymerisation at the parasite apex, after which filaments are translocated by myosin down the length of the parasite pellicle (to the site of the engaged motor complex) carrying with them bound formin. Alternatively,
formin 1 may track with the tight junction during its progression, either in association with the motor complex or independently, where it nucleates actin filaments in situ. The first scenario is reminiscent of a model of formin-mediated activation of actin polymerisation at the cell tip by the fission yeast formin, For3p (Martin and Chang, 2006). In this model For3p is transported along microtubules to the cell tip, where it initiates the nucleation of actin cables that are passed rearwards, some carrying a proportion of the bound formin complex with them. Such a model is particularly attractive given the close association we see between formin 1 and components of the microtubular cytoskeleton in merozoites and tachyzoites (Fig. 2C, 4A-C), and the importance these structures play in apicomplexan cell invasion (Fowler et al., 1998; Morrissette and Sibley, 2002). Attempts underway in our laboratory to monitor active formin localization and its association with nascent actin filaments in live invading cells will be able to distinguish between these alternative hypotheses.

In the absence of a full gene knockout, a definitive demonstration of formin 1 function in malaria parasite invasion will depend on development of a system that can disrupt its activity in merozoites, either by expression of a dominant-negative allele or activation of an inducible knockout. Unfortunately, current methods for tetracycline-induced gene regulation in malaria (Meissner et al., 2005) are not robust, resulting in inconsistent levels of expression, and at this time preclude the investigation of dominant-negative constructs. Furthermore, available vectors for disruption can only carry genes much smaller than either formin (both formins are ~8kb).
The large size of apicomplexan formins, but striking absence of conserved formin autoregulatory domains (Goode and Eck, 2007) raises the potential for identifying novel apicomplexan domains involved in regulation. Furthermore, since Apicomplexa have no orthologues of the small GTPases or other well-characterised upstream effectors of animal or yeast formins (Baum et al., 2006a), the cell factors involved in signalling formin activation in the malaria parasite must involve other, potentially unique, mechanisms. Disruption of these upstream actin effectors should prevent actin polymerisation, thereby inactivating the motor and stopping parasite motility/cell invasion. Significantly, since invasion of erythrocytes is central to malaria parasite pathogenicity, the targeting of such actin regulators may be appropriate for development as drug targets against malaria and other apicomplexan parasites.
Experimental Procedures

All chemicals were from Sigma unless noted otherwise.

*Parasite cell culture and parasite invasion inhibition*

*P. falciparum* (3D7 strain) and *T. gondii* (RH strain) were maintained using standard procedures. *P. falciparum* cultures were grown in human O+ erythrocytes at 4% hematocrit with 0.5% Albumax™ II (Invitrogen). 3D7 is a cloned line derived from NF54, obtained from the late David Walliker at Edinburgh University, UK. *T. gondii* tachyzoites were propagated in human foreskin fibroblasts (HFF) or Vero cells grown in Dulbecco's Modified Eagles Medium (DMEM) with 10% foetal calf serum (GibcoBRL). *P. falciparum* merozoite invasion was captured following treatment with 0.1 μM cytochalasin D to arrest invasion (Miller et al., 1979). Tachyzoite invasion was captured using a recently described potassium shift protocol (Kafsack et al., 2004).

*Recombinant protein expression*

Expression constructs containing FH2 domains with putative FH1 domains for PfFormin1 (nucleotides 6649-7953) and PfFormin2 (nucleotides 6955-8397) and (predicted non-dimerizing) PfFormin1 (nucleotides 6922-7953) and PfFormin2 (nucleotides 7390-8397) FH2 domains alone were generated from 3D7 cDNA and expressed using pGEX4T-1 (Amersham Biosciences). Full length *PfPRF* (amplified from 3D7 cDNA) was expressed as a recombinant hexa-histidine fusion using pProEX-HTb (Invitrogen). All proteins were expressed following transformation into *Escherichia coli* BL21 (DE3) strain and, following
standard purification protocols, used for mouse and rabbit immunization. All primer sequences are available on request.

**Protein purification and preparation for actin polymerisation assays**

GST-formin constructs were purified from bacteria using lysis buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM EDTA pH 8.0, 1 mM DTT and 0.01% NaN₃) supplemented with Complete™ protease inhibitor (Roche Applied Science) as described (Kovar et al., 2003). Peak fractions eluted with 100 mM glutathione from a 1.5 ml glutathione-Sepharose™ column (Amersham Biosciences) were pooled and dialyzed against buffer B50 (10 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA pH 8.0, and 0.01% NaN₃) for 24 h at 4°C. Dialyzed protein was further purified using a 75 ml linear gradient of 50–500 mM NaCl in Source Q-buffer using a 1 ml Source™ 15Q column (Amersham Biosciences). Purified FH2 constructs were dialyzed into storage buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 50 mM NaCl, 10% glycerol, 1 mM DTT, and 0.01% NaN₃) and stored at -80°C. Ca²⁺-actin was purified from an acetone powder of chicken skeletal muscle as described (MacLean-Fletcher and Pollard, 1980) and stored in Ca²⁺-G-Buffer (2 mM Tris-HCl, pH 8.0, 0.5 mM CaCl₂, 1 mM DTT and 0.01% NaN₃). Actin was labelled with either Oregon-green (Invitrogen) or pyrenyliodoacetamide (Invitrogen) as described previously (Kovar et al., 2006; Kovar et al., 2003).

**SDS–PAGE and immunoblot analysis**

*P. falciparum* and *T. gondii* tachyzoite parasite pellets were solubilized in 2x SDS loading buffer and separated on 4-12% (Bis-Tris) or 3-8% (Tris-Acetate) SDS-NuPAGE® gels
(Invitrogen) under reducing conditions. Immunoblotting was performed according to standard protocols. Nitrocellulose membranes (0.45 μM; Schleicher and Schuell) were blocked in 10% (w/v) skim milk, 0.1% Tween 20-PBS for 1 h and probed with primary antisera in 1% milk, 0.1% Tween 20-PBS for 1 h [rabbit anti-PfFormin1 (1:300), rabbit anti-PfFormin2 (1:200), rabbit anti-PfPRF (1:300) and rabbit anti-PfAldolase (Baum et al., 2006b) (1:500)]. Membranes were washed twice in 0.1% milk, 0.1% Tween 20-PBS for 5 min and probed with an appropriate secondary antibodies (Millipore) for 1 h. Following 5 further washes, immunoblots were developed by ECL (Amersham Biosciences).

**Immunofluorescence and transmission electron microscopy**

For light microscopy air-dried smears of *P. falciparum* were fixed for 1 min with 100% methanol at -20 °C, blocked for 30 min in 3% bovine serum albumin (BSA) in PBS, and incubated with primary antisera in 3% BSA/PBS for 1h [rabbit anti-PfFormin1 (1:200); rabbit anti-PfFormin2 (1:300); rabbit anti-PfPRF (1:300); mouse anti-RAP1 (monoclonal 7H8/50 1:200); mouse anti-PfFormin1 (1:50); mouse anti-Actin (monoclonal AC-40, Sigma, 1:100); mouse anti-tubulin (monoclonal DM1A, Sigma, 1:200); mouse anti-PfRON4 (D. Richard and A. F. Cowman manuscript in prep, 1:200)]. Following two 5 min washes in PBS slides were probed with an appropriate secondary antibody (see below). After four further washes, slides were labelled with DAPI nuclear stain (Roche Applied Science) at 0.2 μg ml⁻¹ in Vectorshield® (Vector Labs). Free tachyzoites from culture supernatant or infected host cells were fixed on coverslips in 4% paraformaldehyde (Electron Microscopy Sciences) for 10 min, permeablized in 0.1% Triton X (TX)-100/PBS for 10 min and blocked in 3% BSA/PBS for 1 h. Incubation with primary antisera in 3%
BSA/PBS was for 1h [rabbit anti-PfFormin1 (1:300); rabbit-anti-TgRON4 (Alexander et al., 2005), 1:1000]; rat anti-HA (monoclonal 3F10, Roche Applied Science, 1:500)] followed by four 5 min washes in PBS and incubation with appropriate secondary antibody for 1 h (see below). Following four further 5 min washes, coverslips were mounted with DAPI and Vectorshield®. Secondary antibodies (Invitrogen) for both parasite assays were goat anti-mouse (Alexa Flour-488) and -rabbit (Alexa Flour-594) both at 1:500.

Deoxycholate extraction (25mM) of fixed tachyzoites was carried out in 0.5mM MgCl2-PBS for 40 min at room temperature in a humidified chamber followed by blocking and staining, as described above. Dual colour fluorescence images were captured using a Carl Zeiss Axioskop 2 microscope (Thornwood) with a PCO SensiCam (Motion Engineering Co) and Axiovision 3 software (Carl Zeiss). Movie S1 was captured on a Ziess inverted microscope equipped with AxioVision v4.5 with the raw image stacks deconvolved using the inverse filter algorithm.

Immunolabeling for electron microscopy was performed on 1% glutaraldehyde-fixed schizont or 1% glutaraldehyde, 3% paraformaldehyde-fixed tachyzoite parasite preparations. Schizont sections were a kind gift of S. A. Ralph (University of Melbourne, Victoria, Australia). Tachyzoite parasite preparations were pelleted in low melting agarose, dehydrated with increasing ethanol concentrations, embedded in Lowicryl-White resin (ProSciTech, Australia) and sectioned on a Leica Ultracut R ultramicrotome (Wetzlar). Sections were blocked with 5% BSA and 0.1% Tween 20/PBS and incubated with either rabbit (P. falciparum [1: 20]) or mouse (T. gondii [1:500]) primary antisera against PfFormin1 and subsequently labeled with 25 or 10 nm colloidal gold antibodies (Jackson
ImmunoResearch, Baltimore, USA). Standard staining with 2% aqueous uranyl-acetate and 5% triple lead preceded observation on a Philips CM120 BioTWIN Transmission Electron Microscope.

**Molecular modelling of formin-actin interactions**

Models of the *P. falciparum* formin 1 FH2 dimer were generated by comparative modelling using the MODELLER program (http://salilab.org/modeller/modeller.html), employing the *S. cerevisiae* Bni1p FH2 dimer crystal structure (Xu et al., 2004) (PDB number 1UX5) and the crystal structure of the dimer in association with actin (Otomo et al., 2005) (PDB number 1Y64). The alignment used to generate these structures is shown in Fig S3.

**Actin pull down**

Pull-downs with 100 μg of recombinant FH2, FH1FH2 domains or GST alone and *P. falciparum* schizont-enriched parasite lysate were carried out using a modified published method (Baum et al., 2006b; Jewett and Sibley, 2003). Schizont-enriched cultures ~40 h post invasion, were saponin-lysed and washed extensively to remove contaminating erythrocyte material and extracted in 20 pellet volumes of Buffer A (50 mM KCl, 10 mM HEPES [pH 7.5], 1 mM MgCl2, 1 mM EGTA, 2 mM ATP, and 0.2% Tween20 + protease inhibitor cocktail [Roche Applied Science]). Pre-cleared soluble lysate was incubated with the GST fusion overnight at 4°C and pulled down using glutathione agarose beads. Bound proteins were eluted in 2x sample buffer, boiled and separated by SDS-PAGE. Immunoblots were probed with a mouse monoclonal actin antibody that recognizes PfActin (Field et al., 1993; Patron et al., 2005; Sahoo et al., 2006) (clone C4, Millipore).
Pyrenyl-actin polymerisation assays

Actin polymerisation was investigated by fluorescence spectroscopy in 96-well flat-bottom plate (Corning) by measuring the change in fluorescence intensity (excitation 362 nm, emission 407 nm) with a Gemini XPS microplate reader (Molecular Devices). Pyrenyl-labelled and unlabeled ATP actin monomers were mixed in Ca$^{2+}$-G buffer/0.2 mM ATP to obtain a 12 μM stock solution (4 μM final) of 20% pyrenyl-actin. Pyrenyl-actin stock was converted to Mg$^{2+}$ actin by adding 0.1 volume of 10x ME buffer (10 mM MgCl$_2$, 10 mM EGTA) for 2 min at 25°C in the presence of Antifoam 204 at 5 parts/10$^5$. Varying concentrations of recombinant formin in Mg$^{2+}$-G buffer (as Ca$^{2+}$-G-buffer but with 0.1 mM MgCl$_2$) and 0.1 volume (final reaction volume) of 10x KMEI (500 mM KCl, 10 mM MgCl$_2$, 10 mM EGTA, and 100 mM imidazole, pH 7.0) were assembled separately. The reaction was started by adding the protein-buffer mix to the actin stock, and assayed immediately. Pyrenyl-actin fluorescence was monitored every 5-10 s. Time between mixing of final components and start of fluorometer data collection averaged between 15 and 25 s.

Total internal reflection fluorescence microscopy (TIRFM)

Flow cells were assembled using ESCO Superfrost glass slides (Erie Scientific), cleaned as described (Kuhn and Pollard, 2005), and single 22 x 50 mm coverslips (Fisherbrand) mounted with Parafilm to create a 6 mm flow channel. Prior to TIRFM assays 10-15 μL of 200 nM NEM-myosin from skeletal muscle was passed 1-2 times through flow cells, allowed to adhere to the slide for 1 min and washed twice with 10 μL of HS-TBS (0.6 M NaCl, 50 mM Tris-HCl, pH 7.5) and once with 10 μL of Super-Block (Pierce). These were
stored in a humid chamber and used within 1 h of preparation or kept at 4°C for use within 2 days.

Prior to each assay a fresh flow cell was flushed with 1x TIRF Buffer (9.6 mM imidazole, pH 7.0, 48 mM KCl, 0.96 mM MgCl₂, 0.96 mM EGTA, 48 mM DTT, 0.19 mM ATP, 14.4 mM glucose, 19.2 mg/ml catalase, 96 mg/ml glucose oxidase, 0.48% methylcellulose [4000 cP at 2%], 0.19% BSA). TIRF microscopy assays were performed using Oregon green–labelled ATP actin (30% labelled) converted from Ca²⁺-ATP actin to Mg²⁺-ATP actin by addition of 0.2 volumes of 1 mM EGTA and 0.25 mM MgCl₂. Polymerisation was initiated by adding 2x TIRF Buffer mixed with formin (supplemented with storage buffer (see above) to give appropriate final polypeptide concentration) to the Mg²⁺-ATP actin. The reactions were transferred to a mounted flow cell with imaging initiated as soon as filaments appeared (2–5 min, depending on flow and polymerisation rates). Images of fluorescent actin filaments excited by total internal reflection were collected at 10-15 s intervals with a Hamamatsu C4742–95 CCD camera (Orca-ER) on an Olympus IX-70 microscope. Images were acquired with the Olympus Metamorph Imaging software and processed with IMAGEJ software (National Institute of Health, http://rsb.info.nih.gov/ij).
References


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**Author Contributions**

Experiments were designed and data analysed and interpreted by J.B., C.J.T., A.P., T.D.P. and A.F.C. Parasite transfection, immunoblot and fluorescence microscopy analyses were performed by J.B. and C.J.T. Recombinant protein and biochemical assays (pyrenyl-actin and TIRF microscopy) were performed by J.B. and A.P. Electron microscopy was carried out by M.R., S.B.G and J.B. Molecular modelling of actin-formin interactions were performed and analysed by B.J.S. and J.B. Antibodies against PfRON4 were raised, tested and validated by D.R. The manuscript was written by J.B. T.D.P and A.F.C. All authors discussed the results and commented on the manuscript.

**Figure legends**

Figure 1. *Plasmodium falciparum* expresses two formins. (A) Schematic representation of *S. cerevisiae* formin Bni1p and *P. falciparum* formins. FH2 domain colouring correlates with the structural model (Fig. S3). Numbers below protein schematic indicate amino acid residue derived from reference sequences. Black lines designate constructs used in this study. Expanded sequences upstream of the key tryptophan (W) residue represent putative formin homology (FH)-1 domains with proline (P) residues marked in red. Neither *P. falciparum* formin contain any of the other well-characterized regulatory domains including
the GTPase-binding (GDB) and diaphanous auto-regulatory (DAD) domains found in formins like Bni1p. (B) *P. falciparum* formins are expressed differentially. Immunoblot of whole *P. falciparum* parasite lysate at 8 h intervals across the 48 h lifecycle (0 h = invasion) and whole *T. gondii* parasite lysate (last lane) probed with polyclonal rabbit antiserum raised against recombinant FH2 domains of PfFormin1 and PfFormin2, and recombinant PfPRF (rabbit anti-PfAldolase, loading control). (C) Immunofluorescence and phase contrast micrographs of asexual blood-stage *P. falciparum* parasites stained with the same PfFormin1 or PfFormin2 antisera. Yellow arrows points to periphery of intra-erythrocytic trophozoite. White arrow points to apical pole of merozoite. DAPI nuclear stain. Scale bar = 1 μM.

Figure 2. **PfFormin1 localizes at the apical pole of free merozoites.** Immunofluorescence and phase contrast micrographs of free merozoites post-schizont rupture. Co-localization of rabbit anti-PfFormin1 with: mouse antiseras raised against (A) PfRAP1, a protein found within the apical secretory organelles (the rhoptries) of merozoites, (B) PfGAP45, a key constituent of the invasion motor that localizes to the inner membrane complex but is absent from the extreme apical tip (white arrow); commercial monoclonal antibodies against (C) tubulin and (D) actin. (E) Co-localization of rabbit anti-PfPRF with mouse antiseras against PfFormin1. DAPI nuclear stain. Scale bars = 1 μM. (F) Immunoelectron micrographs showing localization of formin 1 in late schizonts of *P. falciparum* 3D7 with 25-nm-diameter colloidal gold. Labelling is concentrated at the apical pole anterior to the rhoptry body (rh) and neck (rn). Close inspection of the three pellicular membranes (inset) suggests labelling is concentrated at the break in the inner membrane complex (imc)
possibly associated with the supra-alveolar space (sap). Other labels mark the cytosol (cyt) and nucleus (nu).

Figure 3. **Formin 1 follows the moving tight junction during merozoite invasion of the erythrocyte.** Immunofluorescence and phase contrast micrographs of merozoites during invasion in the absence (A) or presence (B) of cytochalasin D (CD) a drug that inhibits actin polymerisation, arresting invasion but not attachment (Miller et al., 1979) (see schematic). Co-localization of polyclonal rabbit anti-PfFormin1 with a monoclonal against PfRON4, a rhoptry neck protein that localizes to the apical pole of merozoites prior to invasion, but follows the tight junction during merozoites invasion (D. Richard and A.F. Cowman *manuscript in prep*). (A) Prior to invasion PfFormin1 begins at the apical pole coincident with PfRON4. (B) In merozoites arrested mid invasion (CD-treated) PfRON4 is clearly in front of PfFormin1, relative to the direction of tight-junction movement (white arrows, black arrow in schematic). Scale bar = 1 μM.

Figure 4. **The *Toxoplasma gondii* formin 1 orthologue localizes to the apical conoid and pellicle of free tachyzoites.** (A) Immunofluorescence and phase contrast micrographs of free *T. gondii* tachyzoites labelled with rabbit PfFormin1 antisera (which recognizes TgFormin1, Fig 1B, S2). TgFormin1 localizes to the tachyzoite periphery and the apically extended conoid (inset, yellow arrow). (B) Co-localization of rabbit anti-PfFormin1 with YFP-α-tubulin (Striepen et al., 2000) in 25mM deoxycholate extracted tachyzoites shows that apical staining of TgFor1 co-localizes with the tubulin-rich conoid (yellow arrow). (C)
Immunoelectron micrographs showing localization of formin 1 in free tachyzoites with 25-nm-diameter colloidal (white arrow heads). imc, inner membrane complex; rh, Rhoptries; apr, apical polar ring; con, remnants of conoid/anterior conoid ring; mtb, sub-pellicular microtubules. (D) Localization of rabbit anti-PfFormin1 in *T. gondii* tachyzoites treated with *Clostridium septicum* α-toxin in cells expressing recombinant TgGAP50-HA (C.J. Tonkin and A.F. Cowman *unpublished data*). TgFormin1 localizes to the supra-alveolar space underlying the outer plasma membrane (plm, closed arrow), distinct from the inner membrane complex (imc, open arrow), highlighted by the location of GAP50 (Gaskins et al., 2004).

Figure 5. **TgFormin1 localizes to the moving junction during tachyzoite invasion of host cells.** Immunofluorescence and phase contrast micrographs of free *T. gondii* tachyzoites arrested mid-invasion by buffer exchange. Co-localization of polyclonal rabbit anti-PfFormin1 with (A) an antibody against TgRON4 (Alexander et al., 2005) and (B) with GAP50. TgFormin1 localizes to the moving junction (white arrows), although some remains at the apical pole as the parasite progresses into the host cell (yellow arrow). (C) Time course of tachyzoite invasion pre-host cell rupture and during invasion, probed with anti-PfFormin1. White arrows (closed) indicate progression of the moving junction during invasion. Yellow arrows mark the apical pole inside the host cell. Open arrows indicate conoid development in the developing daughter tachyzoites. DAPI nuclear stain. Scale bar = 5 μM.
Figure 6. *P. falciparum* formins nucleate actin polymerisation *in vitro*. (A-B) Ribbon diagram models of the two main interfaces between the formin dimer of PfFormin1 and *P. falciparum* actin, based on the crystal structure of yeast Bni1p FH2 domain in complex with actin (Otomo et al., 2005). Details show conservation in amino acid properties at two key interfaces with the actin monomer: (A) hydrophobic residues in the knob region – a domain formed by α helices D and G of a single FH2 monomer; and (B) polar residues in the post-lasso region – a domain formed by the interaction of α helices M-P of one FH2 monomer and the N terminal portion of the second in the FH2 dimer. Formin residue numbers (black) refer to alignment with Bni1p sequence (Fig. S3) whilst actin residues (red) refer to malaria actin sequence (PlasmoDB ID PFL2215w). Residues marked with a diagonal show the PfFormin1 or PfAct residue (above the line) that differ with Bni1p or vertebrate actin (below the line). (C) Recombinant or purified proteins used in this study: GST, *P. falciparum* formins 1 (PfFormin1) and 2 (PfFormin2) FH2 and FH1-FH2 domains; and CSMA, chicken skeletal muscle actin. (D) In vitro pull down of *P. falciparum* actin from parasite lysates by recombinant GST-PfFormin1 and PfFormin2 FH2, FH1FH2 or GST alone. Time courses of actin polymerisation with (E) PfFormin1-FH1FH2 or (F) PfFormin2-FH1FH2 determined by the fluorescence of pyrenyl-actin. Assembly reactions contained 4 μM Mg-ATP actin (20% pyrenyl labelled) alone or in combination with a range of concentrations of formin GST fusion proteins. (G) Nucleation efficiency (the concentration of new ends (Kovar et al., 2003)) of PfFormin1-FH1FH2 (blue circles) and PfFormin2-FH1FH2 (red triangles) as determined at half-maximal polymerisation over a range (E-F) of formin monomer concentrations.
Figure 7. **Total internal reflection fluorescence microscopy (TIRFM) of single actin filaments demonstrates *P. falciparum* formins are barbed-end nucleators.** Nucleation in the presence of Oregon-green labelled chicken skeletal muscle actin reveals different properties for PfFormin1-FH1FH2 and PfFormin2-FH1FH2. (A-C) Sample frames from TIRFM taken at ~300 s (post reaction initiation) with average filament lengths given for 10-20 filaments for (A) 1.5 μM actin alone (33% OG-labelled), (B) 1.5 μM actin with 10 nM PfFormin1-FH1FH2 (inset 0.5 μM) and (C) 1.5 μM actin with 1 μM PfFormin2-FH1FH2. Scale bar = 5 μM. (D-F) Growth of individual actin filament barbed ends observed by TIRFM: (D) actin alone grew at 14.6 ± 0.77 subunits/s (n = 10); (E) actin with 5-10 nM PfFormin1-FH1FH2 grew at 9.0 subunits/s ± 2.3 (n = 20); and (F) actin with 0.2-1 μM PfFormin2-FH1FH2 grew at 8.5 subunits/s ± 3.4 (n = 26).
Supplemental Data

Figure S1. Phylogenetic analysis of apicomplexan formin-like proteins.

Figure S2. Antibodies raised against recombinant *Plasmodium falciparum* formin 1 recognize the orthologous *Toxoplasma gondii* formin 1.

Figure S3. Sequence alignment and molecular modelling of *Plasmodium falciparum* formins.

Supplemental Movies

Movie 1. 3D immunofluorescence microscopy reconstruction of *Toxoplasma gondii* tachyzoite probed using anti-*P. falciparum* formin 1, showing concentration in the parasite pellicle.

Movie 2. Total internal reflection fluorescence microscopy (TIRFM) of actin nucleation using 1.5 μM Actin (300 s, ~x75 speed).

Movie 3. TIRFM of actin nucleation using 1.5 μM Actin with 10nM PfFormin1-FH1FH2 (300 s, x40 speed).

Movie 4. TIRFM of actin nucleation using 1.5 μM Actin with 500nM PfFormin1-FH1FH2 (300 s, ~x75 speed).

Movie 5. TIRFM of actin nucleation using 1.5 μM Actin with 1uM PfFormin2-FH1FH2 (300 s, ~x20 speed).
Baum et al Fig 1

A

ScBn1p (NP_014128)

PFiformin 1 (PFE1545c)

PFiformin 2 (PFL0925w)

B

P. falciparum asexual lifecycle (hrs)

\( \alpha \)Piformin1

\( \alpha \)Piformin2

\( \alpha \)PIPRF

\( \alpha \)PIAld

C

Phase + DAPI

\( \alpha \)Piformin1

\( \alpha \)Piformin2
Baum et al. Fig 3

A

\(\alpha\)PfFormin1  \(\alpha\)PfRON4  +DAPI  Phase  Overlay

B

\(\alpha\)PfFormin1  \(\alpha\)PfRON4  +DAPI  Phase  Overlay
Baum et al Fig 4

A

B

YFP-Tub

\(\alpha\)PfFormin1

+DAPI

Phase

C

D

\(\alpha\)PfFormin1

C. septicum \(\alpha\)-toxin treated

\(\alpha\)PfFormin1

imc

mtb

GAP50-HA

plm

imc

rh

apr

con

mtb
Baum et al Fig 5

A  αPfFormin1  αTgRON4  +DAPI  Phase

B  αPfFormin1  GAP50-HA  Phase

C  Intra-cellular  Extracellular/Invasion (time →)
Baum et al Fig 7

A) Actin

B) +PfFormin1 [10 nm]

C) +PfFormin2 [1 uM]

D) Actin

E) + PfFormin1

F) + PfFormin2

Δ length, subunits x10^3

Δ Time, s

Av. 10.7 ± 0.4 μm

Av. 6.3 ± 1.2 μm

Av. 5.2 ± 0.7 μm

14.6 ± 0.77 subunits/s

9.0 ± 2.3 subunits/s

8.5 ± 3.4 subunits/s

9.0 ± 2.3 subunits/s
Supplemental Data

Formin regulation of actin polymerisation during malaria parasite invasion of the erythrocyte


* these authors contributed equally to this work
Fig. S1. Phylogenetic analysis of apicomplexan formin-like proteins. An unrooted phylogenetic tree of FH2 domains from apicomplexan fromin-like proteins. The consensus tree shown was generated using the neighbor algorithm of PHYLIP

http://evolution.genetics.washington.edu/phylip.html (branch lengths indicate evolutionary distance). Bootstrap values on branches are expressed as a percentage of 1000 replicates and indicate statistical support for groups. Tree shows that apicomplexa all have at least 2 conserved forming-like proteins. Genome accession numbers for each taxon (right) refer to: PF, PV and PY (Plasmodium falciparum, P. vivax and P. yoelii, www.plasmoDB.org), Tg (Toxoplasma gondii, www.toxoDB.org), TA (Theileria annulata, www.geneDB.org) and cdg (Cryptosporidium parvum, www.cryptoDB.org). Genbank accession numbers for Bni1p from Saccharomyces cerevisiae and AFH1 from Arabidopsis thaliana are also listed.
Fig. S2. Antibodies raised against recombinant *Plasmodium falciparum* formin 1 recognize the orthologous *Toxoplasma gondii* formin 1. Immunoblot of whole *T. gondii* parasite lysate taken from wild-type tachyzoites (RH-strain) and tachyzoites expressing TgFormin1 FH1FH2-GFP (under the tubulin promoter) probed with anti-GFP (Roche Diagnostics) and antibodies raised against the recombinant *P. falciparum* formin 1 FH2 domain.
Fig. S3. **Alignment and molecular modelling of Plasmodium falciparum formins.** (A) Alignment of *P. falciparum* formins 1 and 2 with yeast formin Bni1p\(^{S1}\) and human diaphanous-related formin DAAM1\(^{S1}\). Helical regions (determined for Bni1p) are shown above the alignment (N and P are irregular with a 310 pitch) and are coloured from N-terminal (blue) to C-terminal (red). Blue circles indicate residues identified as important in the post/lasso interface between the Bni1p FH2 domain and actin. Red circles similarly indicate residues important for the knob interface of Bni1p FH2 with actin. Residues I1431 and K1601 are essential for Bni1p nucleation function\(^{S2}\). * P. falciparum* formins appear to lack helices F and H. (B) Model of PfFormin1 dimer based on the Bni1p dimer\(^{S2}\). Helices are coloured and numbered as in the alignment. (C) 3D reconstruction of *P. falciparum* actin with PfFormin1 based on the co-crystalization structure of Bni1p with actin\(^{S2}\). Actin monomers coloured red highlight the two major interfaces with the FH2 dimer (post-lasso/actin and knob/actin interfaces). A more detailed view of these interactions is shown in Fig. 6A,B. Other actin monomers in the elongating filament are shaded blue.

**Supplemental References**
