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An exported kinase (FIKK4.2) that mediates virulence-associated changes in *Plasmodium falciparum*-infected red blood cells

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ABSTRACT

Alteration of the adhesive and mechanical properties of red blood cells caused by infection with the malaria parasite *Plasmodium falciparum* underpin both its survival and extreme pathogenicity. A unique family of parasite putative exported kinases, collectively called FIKK (Phenylalanine (F) – Isoleucine (I) – Lysine (K) – Lysine (K)), has recently been implicated in these pathophysiological processes, however, their precise function in *P. falciparum*-infected red blood cells or their likely role in malaria pathogenesis remain unknown. Here, for the first time, we demonstrate that one member of the FIKK family, FIKK4.2, can function as an active kinase and is localised in a novel and distinct compartment of the parasite-infected red blood cell which we have called K-dots. Notably, targeted disruption of the gene encoding FIKK4.2 (*fikk4.2*) dramatically alters the parasite's ability to modify and remodel the red blood cells in which it multiplies. Specifically, red blood cells infected with *fikk4.2* knockout parasites were significantly less rigid and less adhesive when compared with red blood cells infected with normal parasites from which the transgenic clones had been derived, despite expressing similar levels of the major cytoadhesion ligand, *Pf*EMP1, on the red blood cell surface. Notably, these changes were accompanied by dramatically altered knob-structures on infected red blood cells that play a key role in cytoadhesion which is responsible for much of the pathogenesis associated with falciparum malaria. Taken together, our data identifies FIKK4.2 as an important kinase in the pathogenesis of *P. falciparum* malaria and strengthens the attractiveness of FIKK kinases as targets for the development of novel next-generation anti-malaria drugs.

1. Introduction

Malaria caused by *Plasmodium* spp. remains one of the world's most common infectious diseases and a global health priority of utmost importance (Gething et al., 2011; Murray et al., 2012). All of the debilitating and frequently fatal consequences that accompany *Plasmodium falciparum* infection in humans are attributable to replication of the parasite within red blood cells (RBCs) and vary in severity depending on the parasite species and the immune status of the infected individual. In the case of *P. falciparum*, the species responsible for the vast majority of malaria-related deaths, serious complications frequently arise because parasite-infected RBCs (iRBCs) sequester in the microvasculature of various organs (Miller et al., 2002).

Parasite replication within RBCs is accompanied by striking structural and functional alterations. The RBC surface becomes studded with protrusions (called knobs), deformability is reduced and they adhere to vascular endothelium or other RBCs (Cooke et al., 2001, 2004b; Maier et al., 2009). For the parasite, which must maintain the mechanical integrity of the host RBC under the haemodynamic stresses of the host circulatory system and escape destruction by the spleen, these modifications are essential for its survival in vivo. For the infected human on the other hand, obstruction or perturbation of blood flow by iRBCs in the brain or placenta, for example, has serious pathological consequences (Taylor et al., 2004; Desai et al., 2007; Dorovini-Zis et al., 2011).

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At the molecular level, RBC modifications are mediated by up to as many as 400 proteins that are secreted by the parasite and trafficked to various locations in the host RBC (reviewed in (Cooke et al., 2004a; Maier et al., 2009)). *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) is the major adhesion ligand displayed on the iRBC surface and can bind to multiple receptors including CD36, ICAM-1, chondroitin sulphate A (Cooke et al., 2004b) and endothelial protein C receptor (Turner et al., 2013) that are expressed by circulating blood cells, platelets, vasculature endothelial cells and/or placental syncytiotrophoblasts. In addition, numerous other exported parasite proteins also result in major structural and functional changes to iRBCs and play a critical role in malaria pathogenesis (Cooke et al., 2001, 2004b; Maier et al., 2009).

Relatively recent comparative genomic analysis of apicomplexan parasites led to the identification of a novel and unique family of putative parasite kinases, collectively called FIKK, due to the presence of a conserved phenylalanine (F) – isoleucine (I) – lysine (K) – lysine (K) amino acid motif (Ward et al., 2004; Schneider and Mercereau-Puijalon, 2005). FIKKs are characterised by a C-terminal catalytic kinase domain that is distinct from other established eukaryotic protein kinase groups and a variable N-terminal region that does not contain any known functional motifs. Strikingly, the *P. falciparum* genome contains 20 related *fikk* genes, 16 of which are predicted to encode fully functional kinases that are secreted into the host RBC. We and others have recently shown that some of these FIKKs are indeed exported into RBCs and appear to be linked to phosphorylation of distinct components of the host RBC membrane skeleton, however, their function in the biology *P. falciparum* remains completely unknown (Nunes et al., 2007, 2010).

The most unusual member of the FIKK family is FIKK4.2 due to an insertion into the kinase domain of 90 tandemly-arranged copies of a hexapeptide sequence (H – K – S/N – D – N/H/S – N) of unknown function (Fig. 1A). Despite being identified more than two decades ago as a repeat-containing trophozoite antigen (R45) in *P. falciparum* (Bonnefoy et al., 1992) and hypothesised to play a role in RBC remodelling, further characterisation of this protein has never been performed. Here, for the first time, we demonstrate that FIKK4.2 could function as an enzymatically-active kinase in iRBCs and that ablation of its expression in parasites severely impairs the parasite's ability to remodel the host RBC. We also show that the protein is localised in a distinct and novel sub-compartment of the iRBC and is necessary for the formation of morphologically normal and functional knobs on the iRBC surface and their subsequent adhesion to vascular endothelium. Taken together, our data identifies FIKK4.2 as an important additional player in the complex pathogenesis of falciparum malaria and highlights a highly attractive therapeutic target for the development of next-generation anti-malaria drugs.

2. Materials and methods

2.1. Kinase activity

The kinase domain of FIKK4.2 (amino acids 248–1222) is disrupted by blocks of low complexity repeat sequences and the short low complexity downstream sequence (amino acids 403–928) (Fig. 1A). Other *Plasmodium* kinases contain similar internal repeats, albeit much shorter, which have been shown not to interfere with kinase activity (Ward et al., 2004). We synthesised an *Escherichia coli* codon-optimised version of the FIKK4.2 kinase domain lacking the low complexity sequences (FIKK4.2rKD) and ligated it into the expression vector, pCold IV (Clontech Laboratories, Inc., USA), downstream of a thrombin-cleavable His-NusA affinity tag. A second (kinase-dead) version, identical to FIKK4.2rKD but with the catalytic aspartic acid residue at position 264 mutated to alanine (FIKK4.2rKD(D264A)) was also generated. Proteins were expressed separately in *E. coli* (BL21 C41) either in the presence (for activity experiments measured by ADP-Glo™, Promega Corporation, USA) or absence (for [^{32}P] phosphotransfer experiments) of lambda phosphatase and purified by affinity chromatography using Ni-NTA Agarose (QIAGEN GmbH, Germany) followed by size exclusion using Superdex 200 (GE Healthcare Life Sciences, USA). Thrombin cleavage to remove the His-NusA tag from the recombinant proteins was performed using α-thrombin (Sigma–Aldrich, USA) overnight at 4 °C then re-purified by Superdex 200 size exclusion before being used in activity experiments.

Kinase assays were performed using either a conventional [γ-^{32}P]ATP incorporation assay or by ADP-Glo™. For ADP-Glo™, Myelin basic protein (MBP; Sigma–Aldrich) was used as a generic exogenous substrate and reactions performed in 25 μl of kinase buffer (20 mM Tris, pH 7.5, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT). Reactions were started by adding 10 μM ATP and incubated at 30 °C for 30 min. Reactions were stopped by adding 25 μl of ADP-Glo reagent and the percentage of ATP used was measured. Conventional [γ-^{32}P]ATP incorporation was performed as previously described (Dorin-Semblat et al., 2013) using 2 μg of FIKK4.2rKD incubated with either 2 μg MBP, 2 μg calf thymus histones (Sigma–Aldrich) or 2 μg α-casein with 25 μM ATP and 5 μCi [γ-^{32}P]ATP in kinase buffer (20 mM HEPES, 10 mM $MgCl^2$, 20 mM β-glycerolphosphate) for 30 min at 37 °C. Proteins were resolved by 15% SDS–PAGE, stained with Coomassie Brilliant Blue to estimate total protein concentration, then de-stained, dried and [^{32}P]-labelled proteins detected by autoradiography.

2.2. Parasite transfection constructs

FIKK4.2 is transcribed from a single copy, three exon gene (*fikk4.2*, PlasmoDB accession number **PF3D7_0424700**, previously **PFD1175w** or **MAL4P1.230**) located in the sub-telomeric region of *P. falciparum* chromosome 4. To ablate transcription of *fikk4.2* in *P. falciparum* parasites, we generated two independent transfection constructs, pHTKΔ*4.2a* and pHTKΔ*4.2b* (Fig. 2A and B). Each construct consisted of two ~1 kb sequences (F1 and F2) from different regions of *fikk4.2* cloned into plasmid pHHT-TK (Duraisingh et al., 2002). For pHTKΔ*4.2a*, F1a (nucleotides 1–21 of *fikk4.2*) was amplified by PCR using primers TCCCCGCGGTTCTGAATTATTT TTCTTAATAC (mutated nucleotides in bold; wild-type *fikk4.2* sequence is ATGAATTATTTTTCTAAATAC) and GGACTAGTCAATCA GAATATTTCACATTA (nucleotides 978–998 of *fikk4.2*) and cloned into *Sac*II/*Spe*I of pHTK. Similarly, F2a was amplified using CGGAATTCTCACAAAAGTGATCATAAA (nucleotides 2940–2958 of *fikk4.2*) and ACGCTCCTAGGTTAATCTTCATTAATCCACCA (nucleotides 3965–3985 of *fikk4.2*) and cloned into *Eco*RI/*Avr*II of the same plasmid. For pHTKΔ*4.2b*, F1bi was amplified using TCCCCGCGG GTAATTTTCCATTTGAAATATG (nucleotides −563 to −542) and ATTAAGAAAAATAATTCAGAATTAGAAAACTTA (nucleotides −14 to +19); F1bii was amplified using TAAGTTTTCTAATTCTGAATTATTTT TCTTAAT (nucleotides −14 to +19) and GGACTAGTTCATATTTATTG TATATTACT (nucleotides 372–392); F1bi and F1bii were then combined in a subsequent round of PCR to generate F1b (with a mutated, functionally-inactive initiation codon and an in-frame stop) which was cloned into *Sac*II/*Spe*I of pHTK; F2b was amplified using CGGAATTCCTAGTAAATCCATTCAACATT (nucleotides 398–418) and ACGCTCCTAGGGATTATTTTCACTCATATTC (nucleotides 1320–1339) then cloned into *Eco*RI/*Avr*II of the same vector.

2.3. Parasite culture and transfection

Plasmodium falciparum parasites (3D7) were cultured in human RBCs suspended in HEPES-buffered RPMI 1640 supplemented with

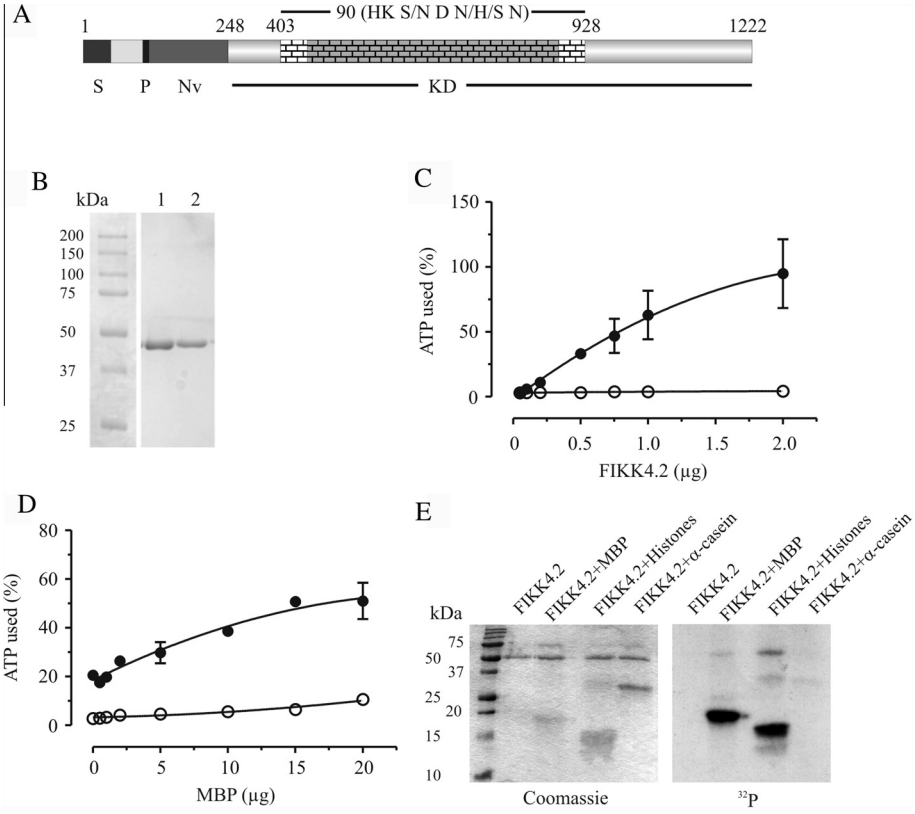

Fig. 1. Features of *Plasmodium falciparum* putative kinase (FIKK4.2) and in vitro kinase activity. (A) Schematic representation of FIKK4.2 indicating salient features typical of the FIKK family of protein kinases. A canonical hydrophobic signal sequence (S) precedes a PEXEL motif (P) and a non-conserved variable region (Nv) followed by a putative kinase domain (KD). FIKK4.2 is an unusual member of the FIKK kinase family since it contains a large block of degenerate repeats (90 (HK S/N D N/H/S N)) (bricked region) that are inserted into the putative KD. (B) Coomassie-stained SDS–PAGE showing purified recombinant His-NusA-FIKK4.2rKD (lane 1) and FIKK4.2rKD(D264A) (lane 2), identical to FIKK4.2rKD but with the catalytic aspartic acid residue at position 264 mutated to alanine. Autophosphorylation activity (C) and kinase activity towards the exogenous substrate, myelin basic protein (MBP) (D) measured by ADP-Glo™ is shown as percentage of ATP used (equivalent to the amount of ADP produced) during the kinase reaction. Autophosphorylation was assayed using increasing amounts of FIKK4.2 (0.1–2 μg). Activity of FIKK4.2 toward MBP was assayed using 0.5 μg of FIKK4.2 and the indicated amounts of MBP (1–20 μg). In both C and D, closed and open circles represent the activity of FIKK4.2rKD or FIKK4.2rKD(D264A), respectively. (E) Kinase activity of FIKK4.2rKD measured by [γ-^{32}P]ATP incorporation using either MBP, calf thymus histones or α-casein as generic substrates. Products of individual phosphorylation reactions were resolved by SDS–PAGE and visualised by staining with Coomassie Brilliant Blue Coomassieor ^{32}P-labelled proteins detected by autoradiography ^{32}P.

0.5% Albumax II as previously described (Cranmer et al., 1997). The knob-positive phenotype was maintained by weekly flotation of parasites in gelatin (Waterkeyn et al., 2001). When necessary, parasites were synchronised at the ring stage using 5% D-sorbitol (Lambros and Vanderberg, 1979). To generate transgenic parasites, 3D7 ring-stage parasites were transfected with 150 μg of plasmid DNA by electroporation and then cultured in the presence of 2.5 nM WR99210 (Sigma–Aldrich) for approximately 30 days until viable parasites were observed in Giemsa-stained blood smears (Wu et al., 1996; Fidock and Wellems, 1997). Parasites were then cultured in the presence of 2.5 nM WR99210 (Sigma–Aldrich) and Ganciclovir (4 μM; Hoffmann La-Roche, USA) to select for double crossover recombinants. Clonal parasite lines were then derived from double-drug-resistant populations by limiting dilution.

2.4. Southern blotting

Genomic DNA (gDNA) was extracted from parasite cultures using Nucleon BACC2 (GE Healthcare Life Sciences) and Southern blotting performed using digoxigenin (DIG)-labelled probes (Hoffmann La-Roche). pHTKΔ*4.2a* and gDNA extracted from clones 1E2, 1G11 and 2H9 was digested with *Hind*III and probed with labelled human dihydrofolate reductase (h*dhfr*) or F2a probes. pHTKΔ*4.2b* and gDNA from clones 3E10, 4F8, 5E3, 6F2 was digested with *Hinc*II and probed with labelled h*dhfr* or F2b.

2.5. Western blotting and immunofluorescence microscopy

Cultured iRBCs were harvested on Percoll, solubilised in reducing Laemmli sample buffer and western blotting performed using standard methods. Immunofluorescence was conducted on thin, air-dried and fixed (9:1 acetone/methanol) culture smears. The anti-FIKK4.2 mouse monoclonal antibody (mAb) (1:1000) was raised against the 11 × HKSDSN-like repeats from the central region of the protein originally identified in clone R23 (Bonnefoy et al., 1992). Rabbit (1:1000) or mouse polyclonal anti-SBP1 (1:500), rabbit polyclonal anti-KAHRP (1:500), rabbit polyclonal anti-VARC (1:100), rabbit polyclonal anti-MESA (1:2000), rabbit polyclonal anti-Pf332 (1:500) and rabbit polyclonal anti-HSP70 (1:1000) were all raised in-house and have previously been described and used by us and others in numerous published studies. Rabbit polyclonal anti-spectrin (1:1000) was from Sigma–Aldrich. Rabbit polyclonal anti-PFB0090c (J-dots) (1:1000) was provided by Dr. Alexander Maier (Australian National University, Canberra, Australia). Primary antibodies were detected by incubation for 1 h with anti-mouse or anti-rabbit IgG conjugated to AlexaFluor488 or AlexaFluor568

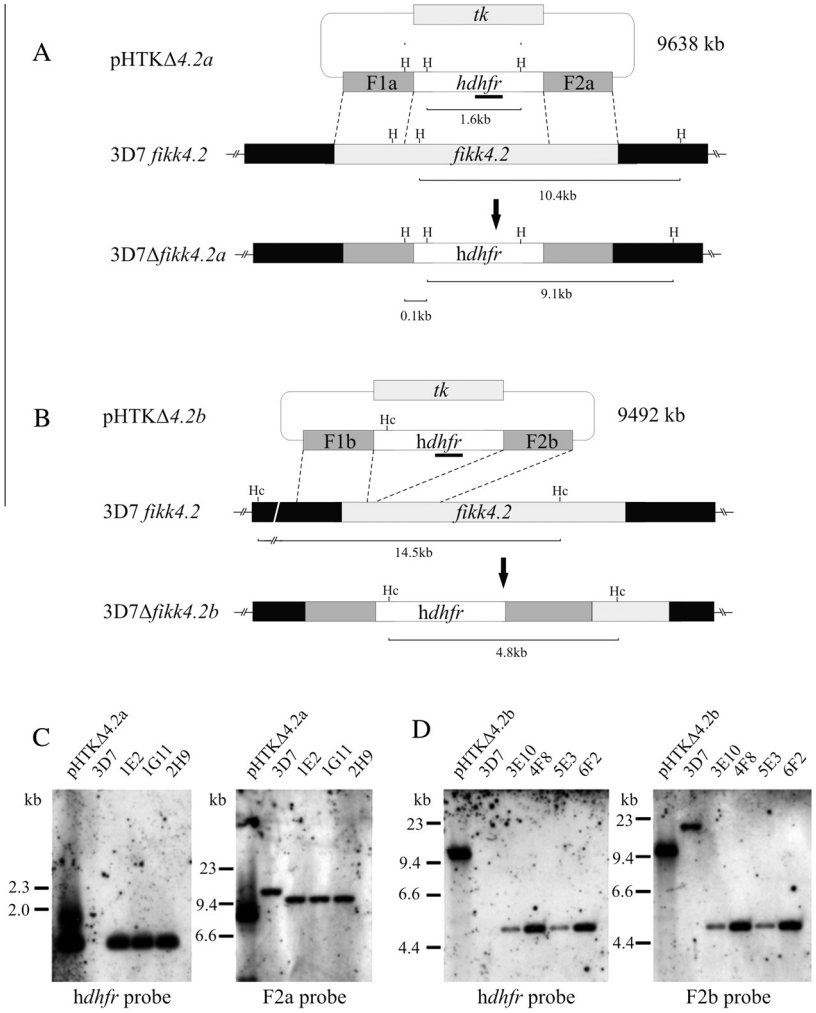

Fig. 2. *fikk4.2* transfection constructs and integration events. (A, B) Schematic representation of the two transfection plasmids (pHTKΔ*4.2a* and pHTKΔ*4.2b*) used to target and disrupt the *fikk4.2* gene in *Plasmodium falciparum* 3D7 parasites (3D7 *fikk4.2*) and the expected double crossover homologous recombination events (3D7Δ*fikk4.2a* and 3D7Δ*fikk4.2b*). The black boxes represent upstream and downstream DNA sequence flanking the *fikk4.2* gene locus. The position of *Hind*III (H) and *Hin*cII (Hc) restriction sites, the predicted length of restriction fragments and the position of the human dihydrofolate reductase (h*dhfr*) probe (thick black line) are shown. (C) Southern blot analysis of *Hind*III digested pHTKΔ*4.2a* and genomic DNA from 3D7 parasites and three different 3D7Δ*fikk4.2a* clones. Hybridisation of h*dhfr* and *fikk4.2*-F2a probes to digested DNA from all 3D7Δ*fikk4.2a* parasite clones revealed restriction fragment sizes consistent with disruption of *fikk4.2* by integration of the h*dhfr* drug-resistance cassette. (D) Southern blot analysis of *Hin*cII digested pHTKΔ*4.2b* and genomic DNA from 3D7 parasites and four different 3D7Δ*fikk4.2b* parasite clones. Hybridisation of h*dhfr* and *fikk4.2*-F2b probes to restriction enzyme-digested DNA from all 3D7Δ*fikk4.2b* parasite clones revealed restriction fragment sizes consistent with disruption of *fikk4.2* by integration of the h*dhfr* drug-resistance cassette.

(1:1000, Molecular Probes, USA). Slides were mounted in Vectashield (Vector Laboratories, USA) containing 2 μg/ml of DAPI and visualised using a fluorescence microscope (BX51; Olympus).

2.6. Measurement of RBC membrane rigidity

Single cell micropipette aspiration was used to quantify the shear elastic modulus (rigidity) of RBC membranes exactly as previously described (Glenister et al., 2002, 2009).

2.7. Quantitation of RBC adhesion

Parasite cultures were synchronised at the ring stage, allowed to mature in culture for 24 h, then adhesion of iRBCs to CD36 expressed on the surface of platelets was quantified under static or flow conditions as previously described (Beeson et al., 1998; Cooke et al., 2002a,b). In each experiment, one or more transgenic parasite clones were compared with the parental 3D7 parasite line from which the transgenic parasites were derived as previously described (Glenister et al., 2009).

2.8. Detection of surface-exposed PfEMP1

In parallel, the amount of PfEMP1 exposed on the surface of iRBCs was detected and quantified using the indirect trypsin cleavage assay exactly as previously described (Cooke et al., 2006).

2.9. Scanning electron and atomic force microscopy

RBCs from synchronised parasite cultures (~5% mature trophozoites) were prepared using standard methods, sputter-coated with gold, and then examined using a Quanta 200 FEG Environmental Scanning Electron Microscope. To quantify knobs on the surface of iRBCs, air-dried thin blood smears were imaged as previously described (Hutchings et al., 2007) using a Dimension 3100 scanning probe atomic force microscope equipped with a NanoScope IIIa controller (Veeco Metrology, USA). Images were

collected in tapping mode in air using a silicon cantilever then processed and analysed using Nanoscope software (Veeco Metrology).

3. Results

3.1. FIKK4.2 kinase activity

Sequence analysis reveals that all FIKK proteins contain potentially active catalytic kinase domains and previous studies have demonstrated that kinase activity can be immunoprecipitated from parasite lysates using FIKK-specific antibodies (Nunes et al., 2007, 2010). First, we therefore sought to determine whether FIKK4.2 demonstrated protein kinase activity in vitro. Using an *E. coli* codon-optimised version of the FIKK4.2 kinase domain devoid of its central repeat region, we expressed and purified the kinase domain of FIKK4.2 to homogeneity (Fig. 1B). When measured by ADP-Glo™ assay, the recombinant FIKK4.2 was able to hydrolyse ATP, both in the absence (Fig. 1C) and presence (Fig. 1D) of MBP (a standard artificial substrate for serine/threonine kinases), with the latter being MBP concentration-dependent. Furthermore, an identical FIKK4.2 recombinant protein, but in which we had mutated the catalytic aspartic acid residue (D264) to alanine, completely abolished enzymatic activity (Fig. 1C and D). Finally, the protein kinase activity of FIKK4.2 was also confirmed using the more conventional approach by clearly demonstrating a selective ability to incorporate radio-labelled phosphate from [γ-^{32}P]ATP into some (MBP and histones) but not all (α-casein) artificial protein substrates (Fig. 1E). Taken together, these data provide strong evidence that FIKK4.2 is a bona fide kinase that is highly likely to be functional in iRBCs.

3.2. Generation of *fikk4.2*-knockout (KO) parasite clones

To investigate the function of *fikk4.2* we generated transgenic *P. falciparum* parasites in which *fikk4.2* was specifically disrupted. We used two different targeting constructs (pHTKΔ*4.2a* (Fig. 2A) and pHTKΔ*4.2b* (Fig. 2B)) and generated a total of seven double-drug-resistant parasite clones by limiting dilution from four independent transfection events of 3D7 parasites. Southern blot analysis of restriction enzyme-digested DNA using gene-specific or h*dhfr*-derived probes confirmed that *fikk4.2* was disrupted as expected in each of the seven clones (Fig. 2C, D). Integration was further confirmed using PCR (data not shown). Under standard culture conditions, although not systematically quantified, all transgenic parasites replicated at rates indistinguishable from the parental 3D7 parasites from which they were derived, demonstrating that *fikk4.2* is dispensable for the *P. falciparum* asexual RBC cycle in vitro.

3.3. Localisation of FIKK4.2

Four transgenic parasite clones (two randomly selected clones derived from each of the two different targeting constructs (1E2, 2H9 and 3E10, 6F2) and parental 3D7 parasites were analysed for FIKK4.2 expression by western blotting using a mAb raised against the central repeats of FIKK4.2, a region of the protein that is unique to FIKK4.2 (Bonnefoy et al., 1992; Schneider and Mercereau-Puijalon, 2005). In RBCs infected with 3D7 parasites, FIKK4.2 was detected as an approximately 200 kDa band (Fig. 3A). Although this is slightly larger than expected based on the predicted molecular weight for this protein of approximately 143 kDa, it is a well described phenomenon that many exported multi-repeat-containing *Plasmodium* antigens migrate in polyacrylamide gels at much larger than their expected molecular weight (Ravetch et al., 1985; Pologe et al., 1987). No reactivity was observed against RBCs infected with any of the four transgenic parasites, confirming that expression of FIKK4.2 had been ablated in all KO clones (Fig. 3A).

Next, the same anti-FIKK4.2 mAb was used in immunofluorescence experiments to localise the protein within iRBCs (Fig. 3B, C). As the localisation of parasite proteins may change throughout the *P. falciparum* lifecycle, tightly synchronised parasites were used as previously described (Lambros and Vanderberg, 1979; Waterkeyn et al., 2001). Specific punctate fluorescence was observed in the cytoplasm of RBCs infected with ring-stage 3D7 parasites. A similar pattern was observed in RBCs infected with trophozoite/schizont-stage parasites, and although the pattern of fluorescence was more diffuse, individual puncta were still clearly discernible. The puncta appeared too small and too numerous to be Maurer's clefts, and did not co-localise with either the classical Maurer's cleft marker SBP1 (Blisnick et al., 2000; Cooke et al., 2006) (Fig. 3B) or the more recently described J-dots (Kulzer et al., 2010) (Fig. 3C). In RBCs infected with very mature parasites, some degree of colocalisation between FIKK4.2 and SBP1 becomes apparent, however, this is most likely due to a generalised compression of all components of the intraerythrocytic compartment at this stage of parasite maturation. The anti-FIKK4.2 antibody showed no reactivity with normal, uninfected RBCs or RBCs infected with any of the four transgenic KO parasite clones, strongly suggesting that the fluorescence observed in parental 3D7 parasites was highly specific for FIKK4.2.

To rule out the possibility that trafficking or localisation of other exported parasite proteins is affected by deletion of FIKK4.2, we compared RBCs infected with parental 3D7 or FIKK4.2 KO parasites that had been labelled with antibodies raised against KAHRP, MESA and Pf332 as three examples of other very well described *P. falciparum*-encoded exported proteins (Coppel et al., 1988; Crabb et al., 1997; Glenister et al., 2009). By comparing immunofluorescence images of iRBCs infected with either 3D7 or FIKK KO parasites, we observed no noticeable differences in the expression or localisation of any of RBC membrane skeleton-associated proteins in RBCs infected with the FIKK4.2 KO parasites, suggesting that trafficking and transport of other exported proteins is not grossly affected by the absence of FIKK4.2 (Fig. 3D).

3.4. Effect of FIKK4.2 on RBC membrane mechanical properties

To determine the contribution of FIKK4.2 to the altered mechanical properties of the membrane skeleton of iRBCs, we measured and compared the shear elastic moduli of normal uninfected RBCs and RBCs infected with either parental, 3D7Δ*fikk4.2a* (1E2) or 3D7Δ*fikk4.2b* (3E10) parasites (Fig. 4A) using a single-cell micropipette aspiration technique. *Plasmodium falciparum* infection dramatically increases the elastic modulus (rigidity) of the RBC membrane and as a result RBCs infected with mature wild-type parasites are approximately threefold more rigid than uninfected RBCs (Glenister et al., 2002). Notably here, RBCs infected with FIKK4.2 KO parasite clones were significantly less rigid than RBCs infected with wild-type 3D7 parasites (mean shear elastic modulus reduced by 21% ($P < 0.05$) and 31% ($P < 0.01$) for clones 1E2 and 3E10, respectively (Dunn's multiple comparison test)). There was no statistically significant difference in the rigidity of the RBC membrane between the two KO clones. Taken together, these data imply that FIKK4.2 contributes significantly to the rigidification of the RBC membrane caused by *P. falciparum* infection.

3.5. Effect of FIKK4.2 on RBC adhesion

Adhesion of iRBCs to vascular endothelium is a hallmark of falciparum malaria infection and is central to pathogenesis. We tested whether disruption of *fikk4.2* affects the adhesive properties of iRBCs under flow conditions that mimic those of the circulatory

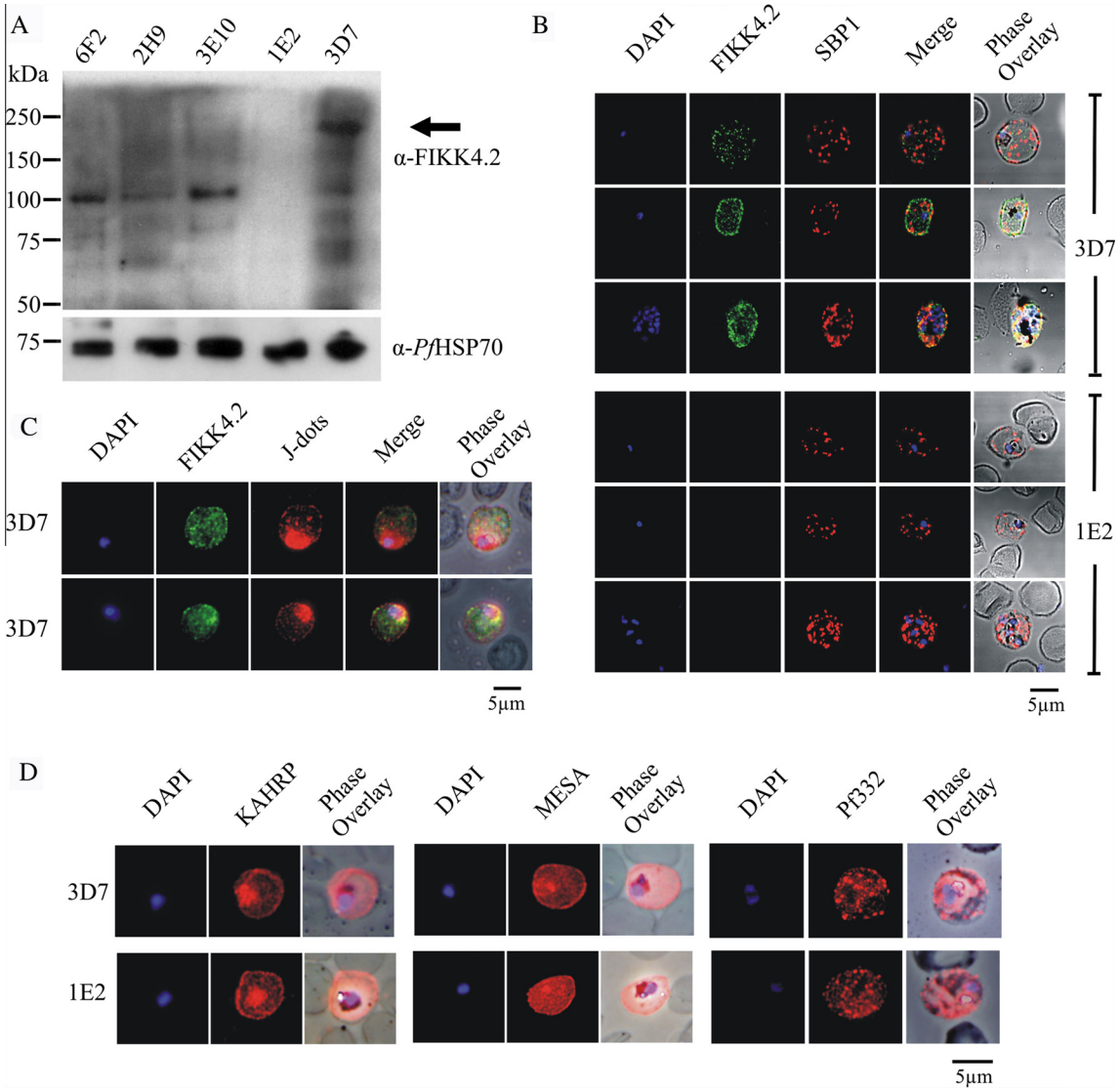

Fig. 3. Expression and localisation of exported proteins in red blood cells infected with parental 3D7 or *fikk4.2* knockout *Plasmodium falciparum* parasites. (A) Red blood cells infected with parental (3D7) or knockout (1E2, 3E10, 2H9 and 6F2) parasites were immunoblotted with α-FIKK4.2 or α-*PfHSP70* (loading control) antibodies. Expression of FIKK4.2 is abolished in all four *fikk4.2* knockout parasite clones (black arrow). (B) Immunofluorescence analysis of red blood cells infected with parental (3D7) or FIKK4.2 knockout (1E2) parasites. Experiments were performed on infected red blood cells that had been synchronised at either the young ring stage or the more mature trophozoite/schizont stage. Air-dried and fixed blood smears on glass slides were dual-labelled with both α-FIKK4.2 and α-SBP1 (Maurer's cleft) antibodies. Merge and phase overlay images demonstrate clearly that FIKK4.2 and SBP1 do not co-localise in infected red blood cells. (C) Immunofluorescence analysis of red blood cells infected with parental 3D7 parasites co-labelled with α-FIKK4.2 and α-PFB0090c (J-dots) antibodies. Merge and phase overlay images indicate that FIKK4.2 and J-dots do not co-localise in infected red blood cells. In all panels, parasite nuclei are counterstained with DAPI. (D) Immunofluorescence analysis of red blood cells infected with either parental 3D7 or FIKK4.2 knockout (1E2) parasites. Infected red blood cells were incubated with antibodies raised against the knob-associated histidine-rich protein (KAHRP), mature-parasite-infected erythrocyte surface antigen (MESA) or *P. falciparum* antigen 332 (Pf332). Parasite nuclei were counterstained with DAPI.

system in vivo using a previously well-described flow-based assay (Cooke et al., 2002a,b). RBCs infected with parental 3D7 parasites bind only to the endothelial cell surface receptor CD36, so we used CD36 expressed on the surface of platelets to quantify adhesion. RBCs infected with each of the four FIKK4.2 KO clones were tested and all consistently bound to CD36-expressing platelets at significantly lower levels than RBCs infected with parental 3D7 parasites (Fig. 4B), with mean levels of adhesion 20–60% lower than 3D7, depending on the individual clone ($P < 0.05$; one sample *t*-test against a theoretical mean of 100% for 3D7).

Since we have previously demonstrated that the level of adhesion of iRBCs under flow can be dramatically different to adhesion under static conditions, and that both static and flow adhesion data when taken together can be highly informative about the mechanism of altered adhesion (Crabb et al., 1997), we also compared the level of adhesion of 3D7 parasites with each of the four FIKK4.2 KO clones to platelet-expressed CD36 in a previously described static adhesion assay (Beeson et al., 1998). In three separate experiments (with three replicates in each experiment), RBCs infected with each of the four FIKK4.2 KO clones consistently bound to CD36-expressing platelets at significantly lower levels than RBCs infected with parental 3D7 parasites (Fig. 4C), with mean levels of adhesion 55–76% lower than 3D7, depending on the individual clone ($P < 0.01$; one sample *t*-test against a theoretical mean of 100% for 3D7). Overall, it appears that adhesion of *FIKK4.2 KO* iRBCs to CD36 is significantly reduced under both static and flow conditions.

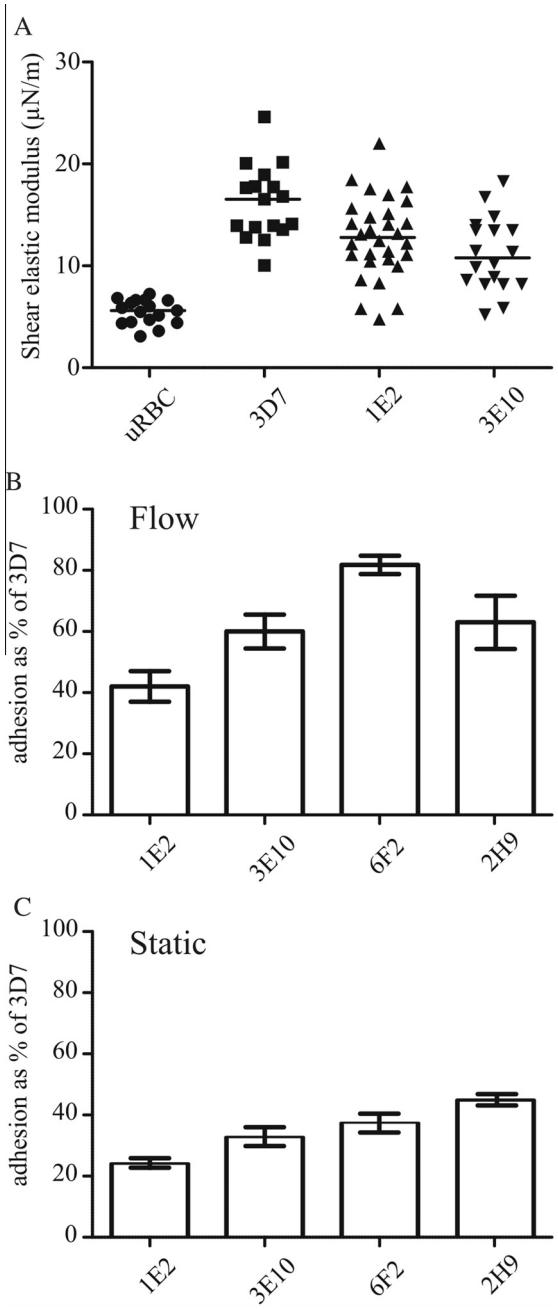

Fig. 4. Disruption of *fikk4.2* alters the mechanical and adhesive properties of *Plasmodium falciparum* infected red blood cells. (A) Membrane shear elastic modulus of normal, uninfected red blood cells and red blood cells infected with wild-type (3D7), 3D7Δ*fikk4.2a* (1E2) or 3D7Δ*fikk4.2b* (3E10) parasites measured by single-cell micropipette aspiration. Each point represents the membrane shear elastic modulus (rigidity) for an individual red blood cell. Horizontal bars represent the median of all data in each group. (B and C) The level of adhesion of red blood cells infected with either wild-type (3D7) or FIKK4.2 knockout parasite clones was compared under either (B) physiologically-relevant flow conditions at a wall shear stress of 0.05 Pa. or (C) in a static (no-flow) adhesion assay. 3D7Δ*fikk4.2a* (1E2 and 2H9) and 3D7Δ*fikk4.2b* (3E10 and 6F2) clones were analysed. Data represent the mean ± S.E.M. (error bars) for three to five independent experiments (performed on separate days) for each of the parasite clones and are expressed as the number of infected red blood cells that adhered for each clone as a percentage of parental 3D7 infected red blood cells. In each experiment, minimum of 500 adherent infected red blood cells were counted.

3.6. Effect of FIKK4.2 on RBC surface-exposed PfEMP1

Defects in iRBC adhesion can be caused by either a reduced abundance or incorrect presentation/anchoring of the major parasite adhesion ligand PfEMP1 on the iRBC surface. In order to determine whether RBCs infected with FIKK4.2 KO parasites displayed altered levels of surface-exposed PfEMP1, we used the previously described indirect trypsin cleavage assay (Waterkeyn et al., 2000; Cooke et al., 2006; Glenister et al., 2009) since there is currently no reliable direct method to quantify PfEMP1 on the surface of iRBCs infected by almost all *P. falciparum* parasite lines. We treated similar amounts of intact, Percol-purified iRBCs at similar parasitemia (>90%) with 100 μg/ml of TPCK-treated trypsin to degrade the extracellular portion of surface-exposed PfEMP1 and then used a specific antibody raised to the C-terminal tail of PfEMP1 (VARC) to detect the presence of the trypsin-insensitive intracellular domain by western blot (Fig. 5). Results for each of the four KO clones tested for adhesion were consistent across at least three biological replicates for each clone, with surface PfEMP1 expressed in all KO parasites as evidenced by the presence of the ~75 kDa trypsin-resistant membrane-spanning cytoplasmic tail of PfEMP1 in the KO lines when compared with parental 3D7 control parasites. Detection of VARC was absent in all non-trypsin-treated samples as expected. Based on the intensity of the band by western blot, it appears that similar amounts of VARC was present on the surface of RBCs infected with at least three of the four KO clones (with the possible exception of clone 1E2, that in this particular experiment, appears to display slightly reduced amounts). We conclude that, overall, RBCs infected with either normal 3D7 or FIKK4.2 KO parasites express similar levels of PfEMP1 on their surface.

3.7. Effect of FIKK4.2 on RBC knobs

Reduced adhesion of FIKK4.2 KO iRBCs under both static and flow conditions in the presence of KAHRP and normal levels of surface-expressed PfEMP1, suggested to us that either the number or arrangement of knobs on the iRBC surface was altered, thus affecting the normal display and function of PfEMP1. We examined RBCs infected with either 3D7 or FIKK4.2 KO parasites by scanning electron microscopy and compared the surface architecture of the iRBCs (Fig. 6). For all four parasite clones examined, RBCs infected with FIKK4.2 KO parasites expressed 3.5- to 6.4-fold fewer knobs than RBCs infected with 3D7 parasites (0.7 ± 0.1 knobs/μm^2 for all KO clones versus 3.2 ± 1.1 knobs/μm^2 for 3D7; mean ± S.E.M.; $P < 0.0001$ by Mann Whitney t test), and appeared abnormally large when compared with knobs on the surface of 3D7-infected RBCs. Further examination of 23–35 randomly selected iRBCs by quantitative atomic force microscopy revealed that although the height of knobs above the RBC membrane on 3D7- or FIKK4.2 KO-iRBCs was not significantly different (22.0 ± 1.2 nm for 3D7

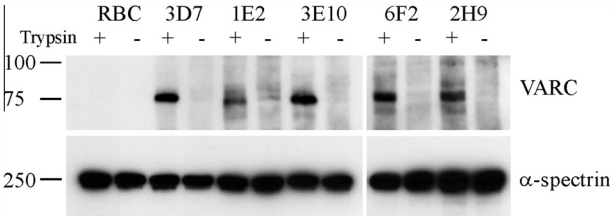

Fig. 5. Red blood cells infected with either 3D7 or *fikk4.2* knockout *Plasmodium falciparum* parasites express similar levels of the major cytoadhesion ligand, PfEMP1, on the red blood cell surface. Western blot analysis of Triton X-100-insoluble/SDS-soluble membrane fractions from infected red blood cells (wild-type (3D7), 3D7Δ*fikk4.2a* (1E2 and 2H9) and 3D7Δ*fikk4.2b* (3E10 and 6F2)), either before (-) or after (+) intact infected red blood cells had been treated with trypsin. Blots were either incubated with α-VARC antibody (to indirectly detect red blood cell surface-exposed PfEMP1) or and α-spectrin antibody (as a red blood cell-loading control). Size of molecular mass markers in kDa is shown. One typical western blot is shown that is representative of at least four performed throughout the course of this study as different combinations of parasite clones were tested.

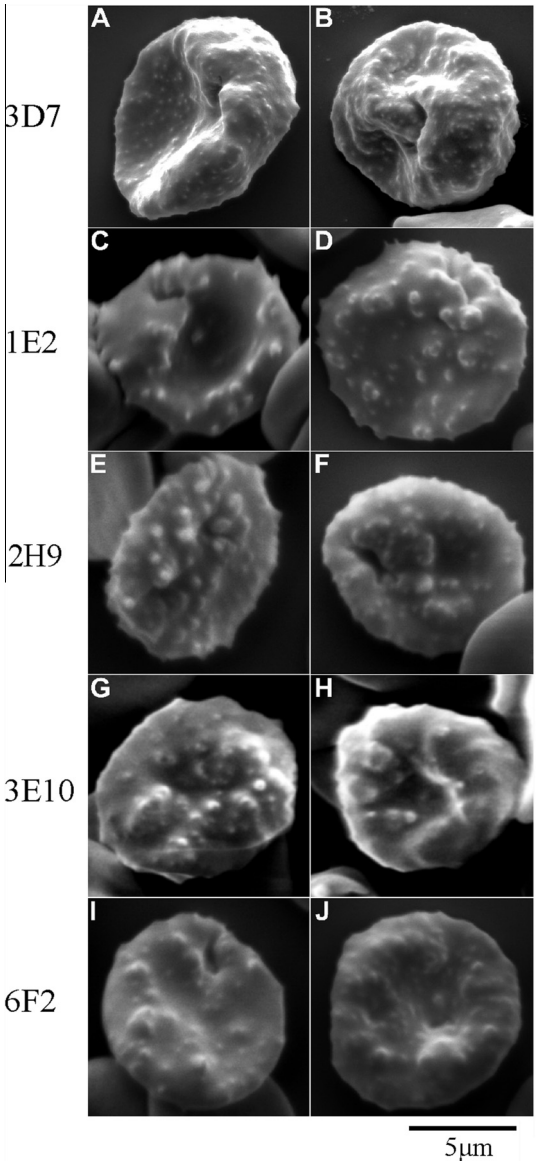

Fig. 6. Knobs are less abundant and abnormally large on the surface of red blood cells infected with FIKK4.2 knockout *Plasmodium falciparum* parasites. Scanning electron micrographs of infected red blood cells are shown. Two red blood cells infected with parental 3D7 parasites (A and B) show normal knob morphology and density when compared with red blood cells infected with each of the four different FIKK4.2 knockout clones (C–J) in which knobs are greatly reduced in number, much larger in size and heterogeneously distributed.

versus 24.8 ± 2.3 nm for all KO clones; mean ± S.E.M.), knobs were 1.3- to 2.0-fold wider in diameter on RBCs infected with FIKK4.2 KO parasites (172.5 ± 10.6 nm for all KO clones versus 97.0 ± 2.4 nm for 3D7; mean ± S.E.M.; $P < 0.0001$ by Mann Whitney *t* test).

4. Discussion

Central to both the survival and pathogenicity of *P. falciparum* is its ability to remodel the RBC in which it resides by secreting numerous proteins (perhaps as many as 400) beyond its own plasma membrane, and selectively trafficking them to various locations in the host RBC (Cooke et al., 2004a; Maier et al., 2009). As mature RBCs lack any endogenous protein trafficking mechanisms of their own, the parasite exports its own specialised machinery into the host RBC cytoplasm to facilitate protein targeting. Secreted proteins are translocated across the parasitophorous vacuolar membrane via a putative *Plasmodium* translocon of exported proteins (PTEX) complex (de Koning-Ward et al., 2009; Riglar et al., 2013) and enter the parasite-induced endomembrane system comprised of Maurer's clefts (flattened discs that become tethered to the RBC membrane (Bhattacharjee et al., 2008; Hanssen et al., 2008)) and various other yet poorly defined vesicle-like structures.

Of the proteins that comprise the exportome of *P. falciparum*, at least 70 are likely to interact with the RBC membrane skeleton and play a role in RBC remodelling (Sargeant et al., 2006). Intriguingly, these include a novel group of putative serine–threonine kinases that until very recently have been almost totally ignored. Furthermore, reversible phosphorylation is a control mechanism for many biological functions including signal transduction and protein trafficking; processes that still remain relatively poorly understood in malaria parasites. The FIKK kinase family has undergone a striking expansion in the *P. falciparum* genome. Whereas most apicomplexan parasites, including most *Plasmodium* spp., possess only a single *fikk* gene, *P. falciparum* has 20 unique *fikks* 16 of which are predicted to encode exported proteins. *Pffikks* are distributed throughout 11 of the 14 chromosomes of the *P. falciparum* genome and are often located in sub-telomeric regions in close association with *var* genes (Ward et al., 2004; Schneider and Mercereau-Puijalon, 2005). A preliminary characterisation of several FIKKs demonstrated that multiple family members are simultaneously expressed and targeted to different locations within the iRBC (Nunes et al., 2007). Taken together, these observations strongly suggest that FIKKs play an important and non-redundant role in the process of iRBC remodelling and thus may have a role in the specific pathogenesis associated with *P. falciparum* malaria.

In our work here, we decided to focus on characterising and determining the function of FIKK4.2 for a number of reasons. Firstly, although the *fikk4.2* gene was in fact first described more than 20 years ago (Bonnefoy et al., 1992), it has been the subject of surprisingly few studies and its function in iRBCs remains enigmatic. Secondly, of the five species of *Plasmodium* parasites known to cause malaria in humans, *fikk4.2* is found only in *P. falciparum*; arguably the most pathogenic species. Thirdly, microarray and RNA-seq analysis show that *fikk4.2* is expressed only in asexual blood-stages of the parasite (no evidence of expression in gametocytes, sporozoites or ookinetes) and levels of expression are maximal in late ring/young trophozoite stages, at the time when other known virulence factors are maximally-expressed and co-incident with the onset of major structural and functional modifications to RBCs. Finally, there is evidence that FIKK4.2 might be involved in protective immunity since it is the target of neutralising antibodies elicited either by natural infection or experimental vaccination (Perraut et al., 2000a,b), despite the fact that FIKK4.2 does not contain a trans-membrane domain and is unlikely to be exposed on the iRBC surface.

Our generation of multiple clones of transgenic parasites, in which expression of *fikk4.2* was ablated, has for the first time, allowed us to perform a detailed functional analysis of this gene. As complementation is still often difficult to achieve in *P. falciparum* (and in this case technically infeasible), we instead chose to engineer multiple independent KO parasite clones based on two different gene targeting strategies – an approach that is widely accepted in malaria research when complementation is not possible (Goldberg et al., 2011). Not surprisingly, disruption of *fikk4.2* had no effect on parasite replication in vitro, as has been previously demonstrated for some other members of the FIKK family as well as numerous other genes that encode exported parasite proteins (Crabb et al., 1997; Cooke et al., 2006; Maier et al., 2007, 2008; Glenister et al., 2009; Nunes et al., 2010). Loss of *fikk4.2*, however, had a significant impact on the parasite's ability to remodel the RBCs in which it resides, which, although dispensable for growth

in culture in vitro, is highly likely to be essential for parasite survival and virulence in vivo. RBCs infected with transgenic parasites in which we specifically inactivated the *fikk4.2* gene displayed significantly reduced rigidification of the RBC membrane and reduced adherence of iRBC to CD36 under both static and physiologically-relevant flow conditions. Notably, although the level of PfEMP1 expression on the surface of RBCs infected with FIKK4.2 KO parasites appeared to be essentially similar to the level expressed by normal wild-type parasites, the number of knobs, which provide the platform for PfEMP1 expression, and their architecture, was dramatically altered. In the absence of FIKK4.2, knobs were less numerous and larger in diameter, taking on a more 'clumped' appearance. Interestingly, such abnormal knob morphology is not unprecedented. For example, Fairhurst et al. (2002) demonstrated that haemoglobin (Hb) CC-containing RBCs infected with *P. falciparum* expressed a reduced number of morphologically-abnormal knobs when compared with normal, Hb AA-containing RBCs and this was also associated with a reduction in their ability to adhere to microvascular endothelial cells under flow in vitro (Fairhurst et al., 2005). Detailed quantitative analysis of the sub-population of aberrant knobs on both HbCC and HbAC RBCs (infected with the 7G8 laboratory-adapted strain of *P. falciparum*) revealed that they were approximately twice the width (120 nm) of those on normal HbAA RBCs (70 nm) (Arie et al., 2005). Interestingly, this is highly consistent with our data here where a reduced number of knobs were also up to twofold wider on RBCs infected with FIKK4.2 KO clones. We therefore interpret our data similarly as indicating a generic influence of FIKK4.2 on cytoadherence due to its contribution to the formation of knobs, with normal morphology and distribution required to permit an appropriate display of functional surface PfEMP1 for efficient adhesion to occur.

We analysed the localisation of FIKK4.2 using mAb raised against the unique central repeats of FIKK4.2 (Schneider and Mercereau-Puijalon, 2005). Although antibodies raised against repeat regions of *P. falciparum* proteins are often cross-reactive, we were able to confirm antibody specificity using our KO parasites. Interestingly, FIKK4.2 was localised in distinct punctuate foci within the iRBC cytoplasm, particularly in younger, ring-stage parasites, which appeared too small and too numerous to be Maurer's clefts, and did not co-localise with the classical Maurer's clefts marker SBP1 (Blisnick et al., 2000; Cooke et al., 2006). Similar vesicle-like structures in the iRBC cytoplasm termed J-dots have recently been described and shown to contain members of both the HSP40 and HSP70 co-chaperone/chaperone family (Kulzer et al., 2010, 2012). While we have clearly ruled out any co-localisation of FIKK4.2 with J-dots, it remains possible that these unique puncta of FIKK4.2, which we now call K-dots, also contain similar, yet unidentified, regulatory proteins that are somehow involved in the transport or correct assembly of proteins necessary for the formation of functional knobs. Clearly more work is necessary to identify whether K-dots are solely packets of FIKK4.2 bound for their final cellular destination and target or whether they are part of a new and yet undescribed complex of proteins. Notably, we have shown that FIKK4.2 is capable of phosphorylating both itself and other (artificial) protein targets, suggesting that it may in fact be part of a more complex regulatory cascade.

In conclusion, we have shown that FIKK4.2 plays an important non-redundant role in parasite-induced modifications of iRBCs that are strongly associated with the virulence and pathogenicity of *P. falciparum*. Protein kinases are generally recognised as attractive, druggable, molecular therapeutic targets and since the FIKK family is unique to apicomplexan parasites, small molecules targeting these enzymes are unlikely to inhibit important members of the human kinome. This makes the FIKK family a highly attractive target for the development of novel, next generation anti-malaria drugs. While we have not yet been able to identify the precise molecular target of FIKK4.2, nor delineate its precise mechanistic role in alteration of the structure and function of RBCs, these remain the holy grail of kinase research and discovery efforts in almost all biological systems. Our current studies underway to generate transgenic parasites expressing epitope-tagged FIKK4.2 chimeras, in combination with advanced phosphoproteomic analyses of iRBCs, may in time reveal the precise cellular target for this unique kinase and further illuminate the precise mechanisms for its biological function.

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