

# *Plasmodium falciparum* signal recognition particle components and anti-parasitic effect of ivermectin in blocking nucleo-cytoplasmic shuttling of SRP

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Signal recognition particle (SRP) is a ubiquitous ribonucleoprotein complex that targets proteins to endoplasmic reticulum (ER) in eukaryotes. Here we report that *Plasmodium falciparum* SRP is composed of six polypeptides; SRP9, SRP14, SRP19, SRP54, SRP68 and SRP72 and a 303nt long SRP RNA. We generated four transgenic parasite lines expressing SRP-GFP chimeric proteins and co-localization studies showed the nucleo-cytoplasmic localization for these proteins. The evaluation of the effect of known SRP and nuclear import/export inhibitors on *P. falciparum* revealed that ivermectin, an inhibitor of importin  $\alpha/\beta$  mediated nuclear import inhibited the nuclear import of PfSRP polypeptides at submicromolar concentration, thereby killing the parasites. These findings provide insights into dynamic structure of *P. falciparum* SRP and also raise the possibility that ivermectin could be used in combination with other antimalarial agents to control the disease.

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**Subject Category:** Experimental Medicine

The protein targeting in *Plasmodium falciparum* is a complex process that involves an extensive network of rough endoplasmic reticulum (ER), an unstacked Golgi situated anterior to the nucleus along with characteristic apicomplexan organelles—rhoptries, micronemes and dense granules.<sup>1–4</sup> The biogenesis of the secretory organelles, their relationship to the organelles of higher eukaryotes and various sorting events that allow the proteins to get targeted to the right place are poorly understood in *P. falciparum*. The signal recognition particle (SRP), a cytoplasmic ribonucleoprotein complex, coordinates the targeting of nascent secretory as well as membrane proteins to the translocation machinery of the cells.<sup>5,6</sup> In addition to the targeting function, SRP also does the elongation arrest or pausing function.<sup>7</sup> SRPs have been identified from all the three kingdoms based on their phylogenetically conserved sequences and their structures.<sup>8</sup> The eukaryotic SRP is composed of a ~300 nucleotide 7S RNA to which six distinct polypeptides; SRP9, 14, 19, 54, 68 and 72 are attached.<sup>8,9</sup> SRPs of bacteria are far simpler than its eukaryotic counterpart. In *Escherichia coli*, SRP consists of a 4.5S RNA to which a single polypeptide, the SRP54 homologue Ffh is attached. SRPs of *Leishmania major*, *Giardia lamblia* and *Trypanosoma brucei* contain SRP19, 54, 68 and 72 homologues but do not possess SRP9/14 homologues.<sup>10,11</sup> The molecular and structural studies in mammalian cells have shown that SRP polypeptides; SRP-9, -14, -19, -68 and -72 are imported into the nucleus where they bind SRP RNA.<sup>12</sup> The partially assembled SRP is exported

out of nucleus in the cytoplasm and is joined by SRP54. The assembled SRP thereafter recognizes the under synthesized polypeptide resulting in elongation arrest.<sup>7</sup> It has been shown that the binding of SRP19 to 7S RNA introduces some conformational changes in the RNA molecule, which enables SRP54 to bind it.<sup>13,14</sup> SRP54 acts as a front runner protein in recognizing the signal sequence flaunted by newly synthesized polypeptide chain of translating ribosome. The phenomenon of elongation arrest is considered to be essential, as the cell has to keep pace with the limited number of SRP receptors available on the membrane. The elongation resumes only when the whole conglomeration is transferred to the Sec62 translocon. The importance of components of this protein translocation machinery has been well documented in humans, where mutations in the components of transport machinery have been shown to cause various human diseases, thereby suggesting that this is an important and vital pathway.<sup>15</sup>

In *P. falciparum*, although a number of studies have reported the identification of few major components of protein translocation pathway by *in silico* analysis using Plasmodb data base,<sup>16–18</sup> however, till now *Plasmodium* protein translocation machinery has not been characterized. In the present study, we identified seven *P. falciparum* SRP constituents and characterized them biochemically as well as for their sub-cellular distribution at asexual blood stages by generating SRP-GFP transgenic parasite lines. We further investigated the effects of exportin/importin and SRP

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**Abbreviations:** AFB1, aflatoxin B1; CQ, chloroquine; ER, endoplasmic reticulum; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; HDP, heme detoxification protein; IVM, ivermectin; LMB, leptomycin B; SRP, signal recognition particle

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assembly inhibitors on parasite growth *in vitro* and studied in detail the mode of action of ivermectin. Our results provide new insights into the molecular organization of *P. falciparum* SRP and set the stage to further analyze the antimalarial effect of ivermectin clinically.

## Results

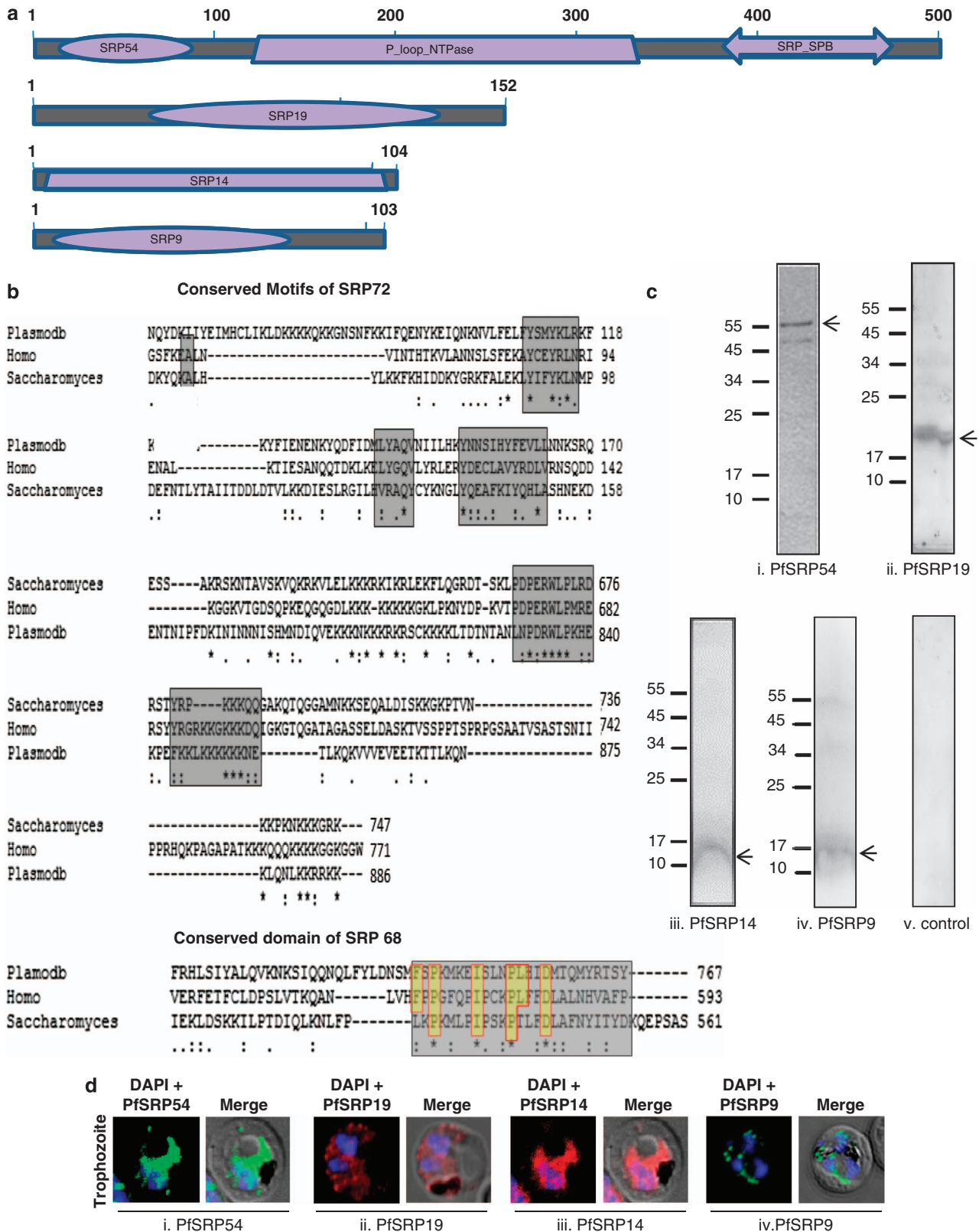
**Identification of *P. falciparum* SRP components, characterization of PfSRP RNA and PfSRP 9, 14, 19, and 54.** The domain specific search of the recent *P. falciparum* data base using the amino acid sequences of the human SRP proteins and SRP RNA was performed. The query identified all the predicted human SRP homologues in *P. falciparum* genome with PlasmoDB numbers PF3D7\_0729000 (PfSRP9), PF3D7\_1203200 (PfSRP14), PF3D7\_1216300 (PfSRP19), PF3D7\_1450100 (PfSRP54), PF3D7\_0621900 (PfSRP68), PF3D7\_1136400 (PfSRP72) and (PfSRP RNA) (Figures 1a and b). PfSRP72 as well as PfSRP68 homologs were identified based on protein–protein and protein–RNA interaction motifs. The annotated PfSRP68 sequence possesses a conserved SRP72 interacting domain, FSPKMKEISLNPLHIDMTQ MYRSTSY of the form FSPKMKEISLNPLHIDMTQMYR [TSYL] located at amino acid positions 728–753 in *P. falciparum*, which harbours the conserved aspartic acid shown to be important for binding of human SRP68 protein.<sup>19</sup> PfSRP72 possesses a conserved 7SL RNA binding domain, PDPERWLPLRD of the form PDRWLPKHEK located at amino acid positions 832–840 in *P. falciparum*. PfSRP72 also possesses the conserved tetrapeptide repeat motif (TPR) consisting of Y110, Q145 and Y153 which has been suggested to mediate interaction with SRP68 (Figure 1b). Together these results suggest that *P. falciparum* SRP is composed of a ~300nt RNA and six polypeptides with masses 12.1, 11.8, 18, 55.9, 92.5 and 107.1 kDa. The coding sequences of predicted PfSRP54, PfSRP19, PfSRP14, PfSRP9 and PfSRP RNA were PCR amplified, cloned and sequenced. The sequence analysis of the PCR products showed no differences from the sequences in PlasmoDB data base.

**Expression and purification of recombinant PfSRP 9, 14, 19 and 54.** To functionally characterize PfSRP polypeptide homologues, these genes were sub-cloned in *E. coli* expression vectors pET28a or pET28b. A moderate level of expression was seen for the four recombinant PfSRP proteins. The recombinant proteins were subsequently purified on a Ni-NTA<sup>+</sup> column under non-denaturing conditions. The apparent molecular masses of the recombinant proteins PfSRP19, PfSRP14 and PfSRP9 are ~19, ~14 and ~12 kDa respectively (Supplementary Figures S1b–d). In the case of PfSRP54, two additional bands of lower molecular weight were always seen along with the intact protein after purification and all three bands were recognized on a western blot by anti-His antibody, thereby suggesting that these two additional bands were the degradation product of the intact protein (Supplementary Figure S1a, iii). The purified recombinant PfSRP54 was unstable as no intact protein was detected after storage. Therefore, to

characterize PfSRP54, two distinct domains covering the entire sequence of PfSRP54; a GTP binding domain (NG domain) and a methionine rich domain (M domain) were cloned in pET28a and expressed. Both the PfSRP54 domains; PfSRP-54<sub>NG</sub> and PfSRP-54<sub>M</sub> were purified on a Ni-NTA<sup>+</sup> column up to near homogeneity (Supplementary Figures S1a, i and ii). The purified recombinant PfSRP proteins were used to raise antibodies in mice and rats.

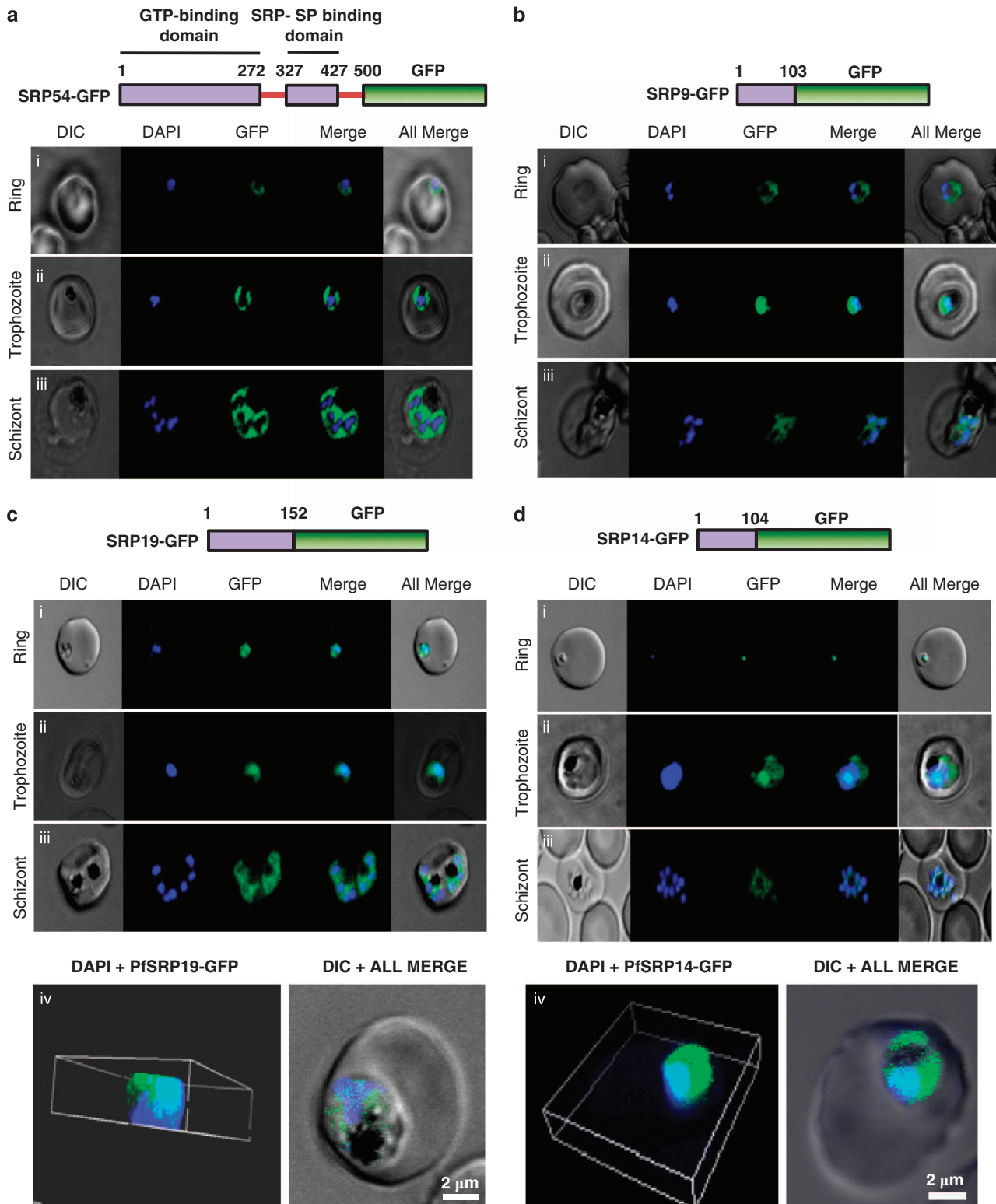
Immunoblot analysis of *P. falciparum* 3D7 parasite lysate using anti-PfSRP54, -PfSRP19, -PfSRP14 and -PfSRP9 antibodies recognized their respective native proteins of ~54, ~19, ~12, and ~12 kDa corresponding to the actual size of the native proteins. Pre-immune sera failed to detect any band in *P. falciparum* lysate (Figures 1c, i–v). The specificity of the antibodies was further confirmed by immunolocalization study at asexual blood stages of the parasite. *P. falciparum* SRP polypeptides are expressed in all three blood stages; ring, trophozoite and schizont (Figures 1d (i–iv) and Supplementary Figures S1e, i–iv). The staining was seen in the parasite cytoplasm and to some extent in the nucleus as well. No staining was observed in parasites with preimmune sera.

**PfSRP polypeptides show bi-compartmental (nucleo-cytoplasmic) localization.** To decipher the sub-cellular localization of *P. falciparum* SRP polypeptides, four transgenic parasite lines expressing PfSRP54, PfSRP19, PfSRP14 and PfSRP9 as chimeric proteins with GFP were generated. Figures 2a–d, upper panels show the schematic of the PfSRP54-GFP, PfSRP19-GFP, PfSRP14-GFP and PfSRP9-GFP fusion constructs used for transfecting the parasite. The localizations of PfSRPs-GFP by fluorescence microscopy of live parasites were also investigated. At the early stage of development >20 h (ring stage), a crescent shaped staining was observed that changed into a ring of fluorescence around the nuclear envelop at the trophozoite stage (Figures 2a–d, lower panels i and ii). In later stages, staining was quite intense and spread through-out the cytoplasm (Figures 2a–d, lower panel iii). At schizont stage, extensive ER branching creating a mesh-like network in some sections was observed (Figures 2a–d, lower panel iii). The PfSRP54 staining was mainly observed in cytosol (Figure 2a, lower panels). In comparison to PfSRP54 distribution in the nucleus, staining for PfSRP19, PfSRP14 and PfSRP9 in subnuclear areas was quite intense at the asexual blood stages particularly at the trophozoite stage (Figures 2b–d, lower panel ii, Supplementary Figure S2). The inspection of transgenic parasites by confocal microscopy (mid-depth Z slice) also revealed the nucleo-cytoplasmic distribution of PfSRP-chimeric proteins (Figures 2c and d, lower panel iv). To further confirm the bi-compartmental localization of PfSRP polypeptides, co-localization experiments for the parasite SRP polypeptides with PfNOP1, a known nucleolar marker<sup>20</sup> or with PfBiP, an ER marker<sup>21</sup> were performed with their respective antibodies. A considerable overlap in staining was observed between three PfSRP polypeptides; PfSRP19, -14, and -9 with PfNOP1 and with PfBiP protein (Figures 3a and b, i–iv). Importantly, considerable co-localization of PfSRP54 with PfNOP1 in early stages of parasite development i.e. merozoite and ring stages (Figure 3a, i and Supplementary Figure S3)

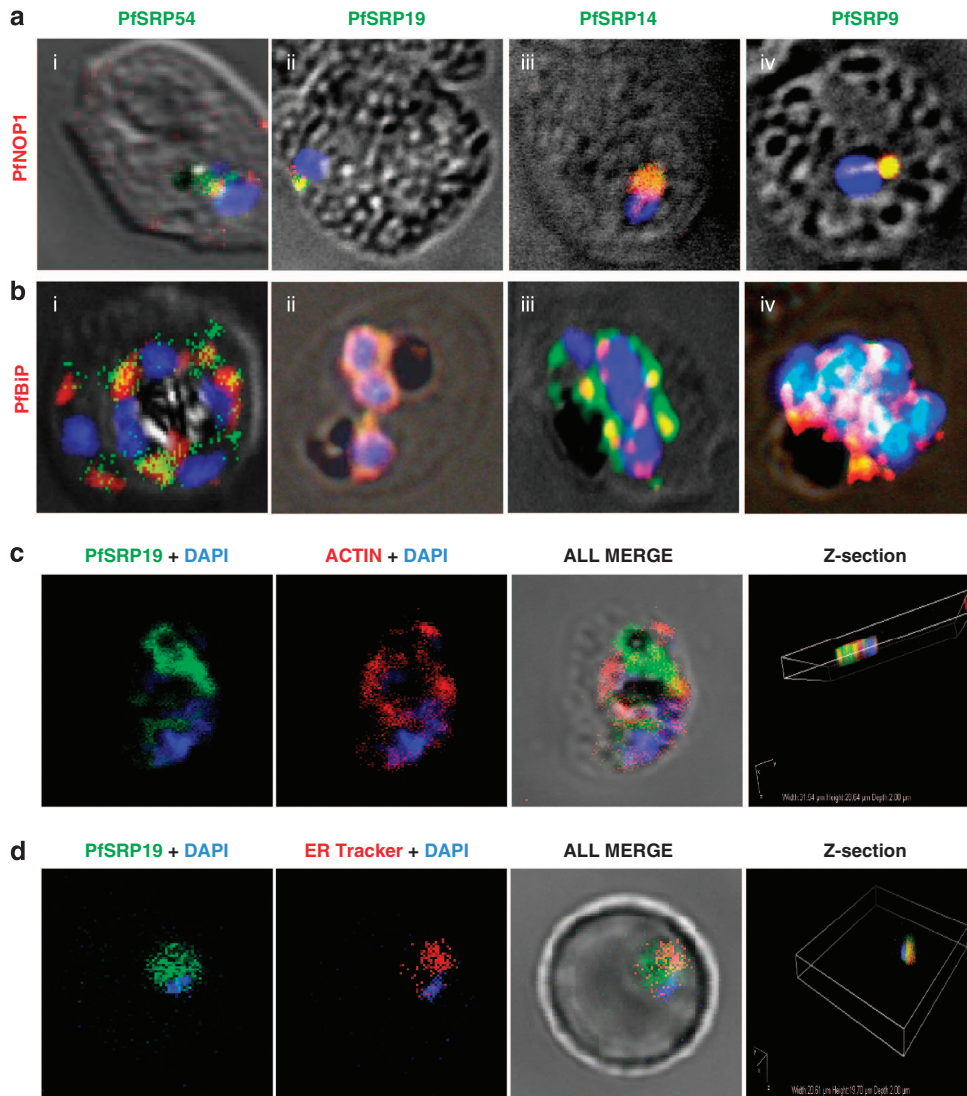


**Figure 1** Schematic representation of domain architecture and expression of PfSRP polypeptides. (a) conserved domains of PfSRP54, PfSRP19, PfSRP14 and PfSRP9 predicted by CDART (<http://www.ncbi.nlm.nih.gov/cdd>). (b) Aligned representation of conserved domains of PfSRP72 and PfSRP68. Regions marked in yellow show conserved sequences. (c, i-iv) Western blot of expression of PfSRP54, PfSRP19, PfSRP14 and PfSRP9 in *P. falciparum* as detected by specific anti-PfSRP54, anti-PfSRP19 and anti-PfSRP14 and PfSRP9 sera in the parasite lysate. (c v) Western blot with mice pre-immune sera (d) Immuno-fluorescence staining of PfSRP54, PfSRP19, PfSRP14 and PfSRP9 in *P. falciparum* detected by specific anti-PfSRP54, anti-PfSRP19 and anti-PfSRP14 and PfSRP9 sera





**Figure 2** Localisation of GFP fused PfSRP54, PfSRP19, PfSRP14 and PfSRP9 polypeptides. Upper panels of **a**, **b**, **c** and **d** show schematic representation of respective GFP fused PfSRP polypeptides. Lower panels show the expression of PfSRP polypeptides in three asexual blood stages of *P. falciparum* i.e. ring, trophozoite and schizont stage. Panel **c(iv)** and **d(iv)** show three dimensional reconstruction of confocal Z-stack merged images of GFP fused PfSRP19 and PfSRP14 (green), respectively, along with nuclear stain DAPI (blue)



**Figure 3** Co-staining of PfSRP polypeptides with nucleolar marker PfNOP1, ER marker PfBiP and cytoplasmic marker Pfactin. (a, b and c) *P. falciparum* infected erythrocytes stained with anti-PfSRP antibodies (green), anti-PfNOP1 (red), anti-PfBiP (red) and anti-Pfactin (red) respectively. (d) localization of GFP fused SRP19 (green) with ER tracker (red). DAPI is used for nuclear staining (blue)

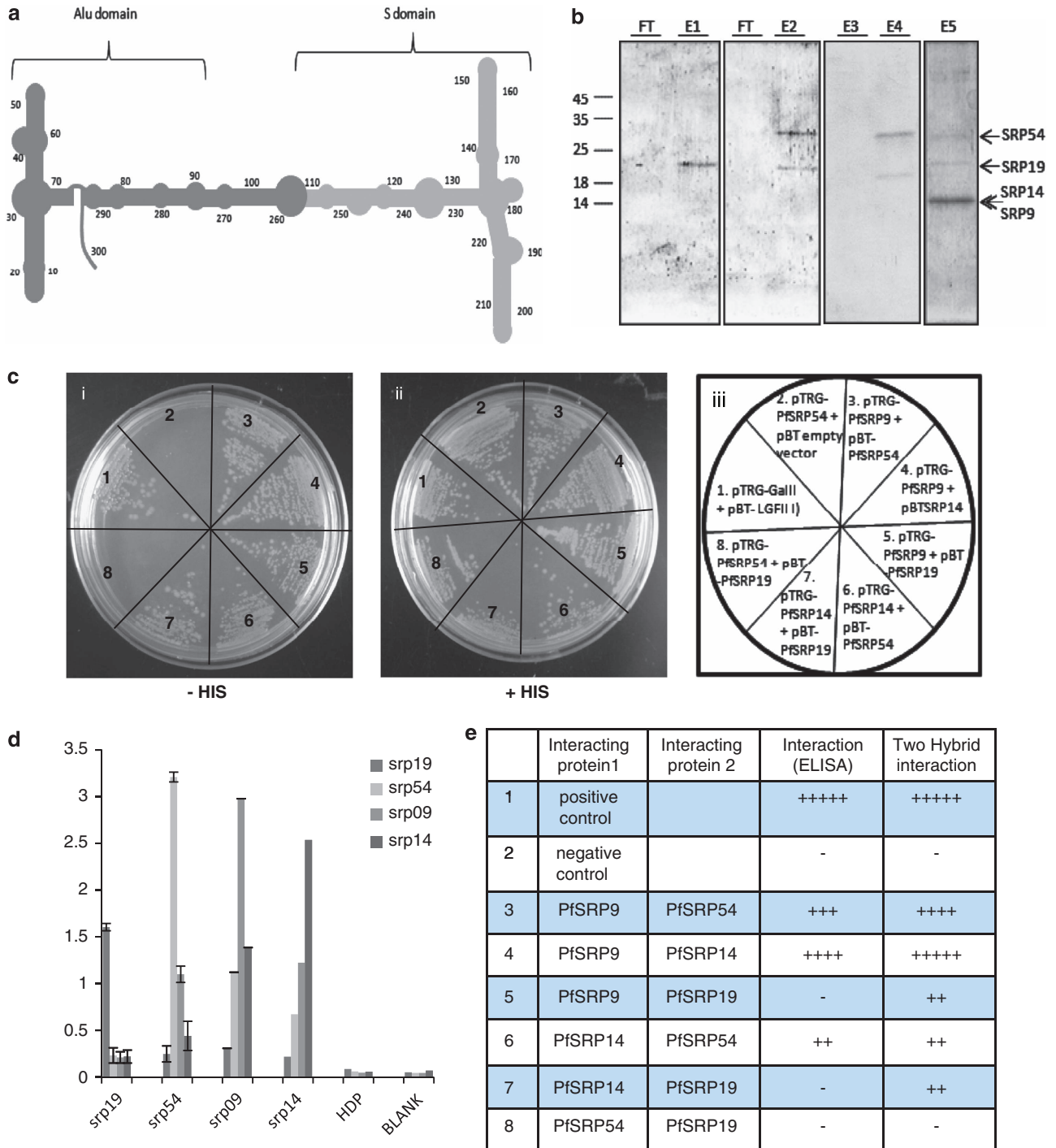
was observed. In addition considerable co-localization between the GFP protein and the cytoplasmic marker Pfactin, along with ER tracker (invitrogen) was also observed (Figures 3c and d). These results collectively suggest that the parasite's SRPs reside in nucleus and ER membrane.

**PfSRP assembly involves protein–RNA and protein–protein interactions.** *In vitro* binding studies in mammalian cells have revealed that SRP RNA is at the centre of structure of SRP complex.<sup>9</sup> Protein–RNA interaction by an RNA binding assay and protein–protein interactions by bacterial two hybrid analysis were performed with PfSRP RNA and between PfSRP proteins to understand the assembly of *P. falciparum* signal recognition particle.

The results of protein/RNA interaction studies showed that PfSRP19 bound to the S domain of PfSRP RNA (Figure 4a) as

well as to the full length SRP RNA (Figure 4b, lane E1). Similarly, PfSRP54 also bound the S domain and intact PfSRP RNA similar to PfSRP19 protein (Figure 4b, lanes E2 and E4). Moreover, PfSRP-9, -14, -19 and -54 together bound strongly to the intact full length PfSRP RNA (Figure 4b, lane E5). However, PfSRP RNA was unable to bind to a non-SRP protein, PfHDP (25 kDa) (Figure 4b, lane E3). These results support identification of PfSRP genes in the present study and further suggest that PfSRP RNA like its other homologs provides a backbone for the coordinated assembly of PfSRP proteins.

To know whether PfSRP assembly also involves protein–protein interactions besides protein–RNA interactions, direct interaction studies between PfSRP54, -19, -14 and -9 using an ELISA based approach or by a well established bacterial two-hybrid interaction approach were performed.



**Figure 4** Interaction between PfSRP polypeptides and SRPRNA. (a) schematic diagram of secondary structure of PfSRP RNA showing the Alu domain and S domain (b). Binding of SRP RNA to SRP 19 and M domain of SRP 54. FT represents the lanes with flow through. Elutes of S domain of SRP RNA binding (by DEAE sepharose method) showing the presence of SRP19 (E1), SRP19 and M-domain of SRP54 (E2). Elutes of full SRP RNA binding (by DEAE sepharose method) with non-SRP protein PfHDP (E3) and PfSRP19 and SRP54 (E4), and all four PfSRP54 (M-domain), PfSRP19, PfSRP14, PfSRP9 (E5). (c) Interaction between protein components of PfSRP polypeptides using Bacterial Two Hybrid system. Growth of cotransformed reporter strain XL1-blue in dual selective (i) and non-selective (ii) medium, (iii) shows the plasmid constructs used to cotransform the reporter strain. (d) Interaction between protein components of PfSRP polypeptides using ELISA. X-axis shows the coated protein and Y-axis is absorbance read at 490 nm. (e) Table showing summary of interactions among all PfSRP polypeptides using both ELISA and Bacterial Two Hybrid system

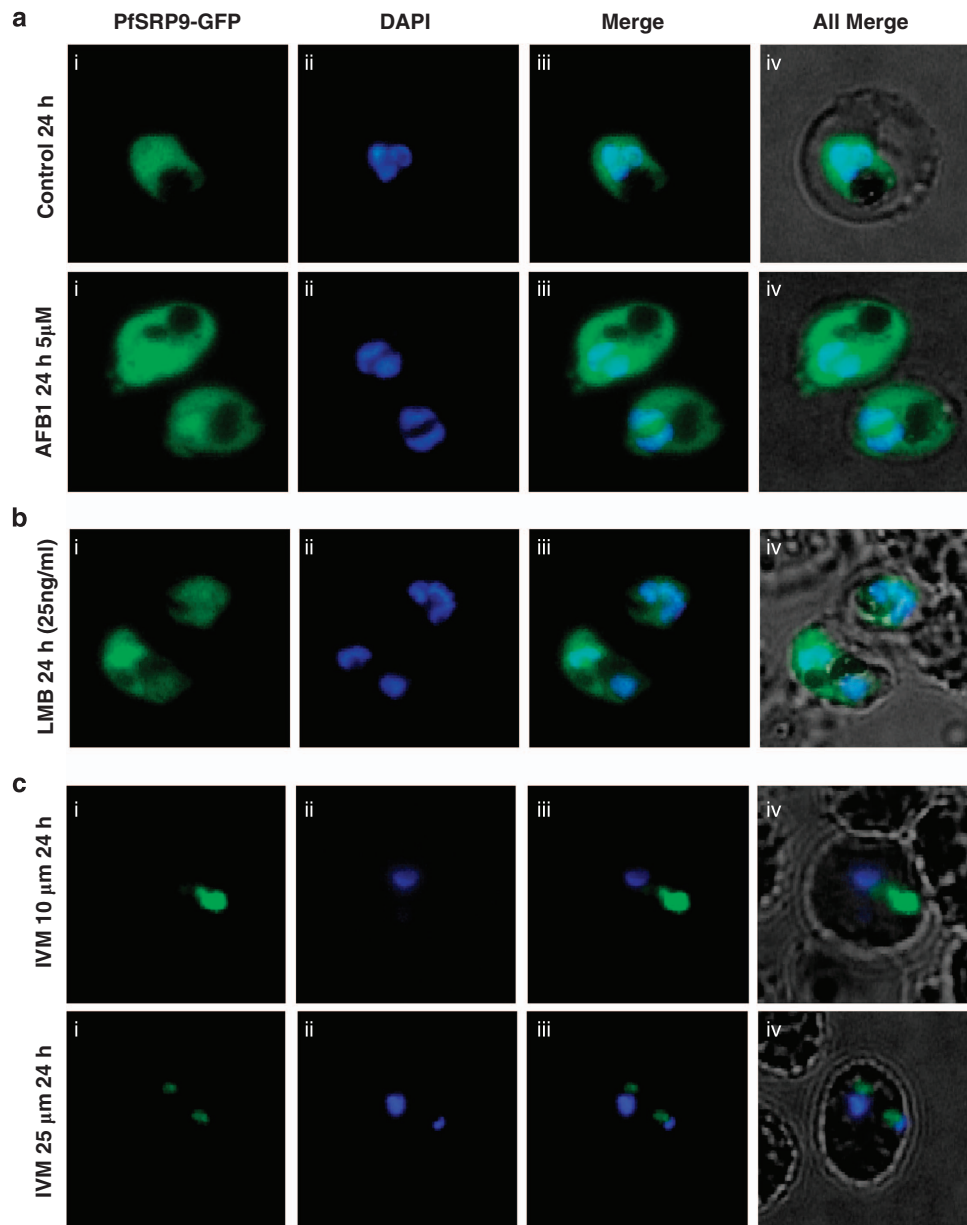
Varying degrees of interactions among the four SRP polypeptides were observed but no interaction between PfSRP19 and PfSRP54 was seen (Figures 4c–e and

Supplementary Figure S4). The associations between these PfSRP proteins *in vivo* were also illustrated by the co-localization studies performed at asexual blood

stages of the parasite using their respective antibodies (Supplementary Figure S2).

**Effect of aflatoxin B<sub>1</sub>, leptomycin B and ivermectin on PfSRP distribution and parasite growth.** A number of inhibitors/drugs such as aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), leptomycin B (LMB) and ivermectin have been shown to either interact with SRP components or inhibit protein nuclear export/import machinery.<sup>22–24</sup> We tested the effect of these inhibitors on transgenic parasite lines. AFB<sub>1</sub> up to 5  $\mu$ M and LMB up to 100 ng/ml had no significant effect on PfSRP9-GFP protein distribution and on parasite growth (Figures 5a and b and

Supplementary Figures S5a and b). Ivermectin, an inhibitor of importin  $\alpha/\beta$ , at 10  $\mu$ M to 25  $\mu$ M showed pronounced effects 24 h after the treatment on PfSRP9-GFP transgenic parasites (Figure 5c). The distribution of GFP-fused PfSRP9 chimeric protein was concentrated in parasite cytoplasm in the treated parasites, in comparison to the untreated parasite culture where the chimeric protein showed the nucleo-cytoplasmic distribution. The effect was more pronounced at 25  $\mu$ M concentration of ivermectin (Figure 5c). The removal of ivermectin from the culture media after 24 h did not alter the growth of the parasite i.e. effect of the drug was irreversible. A similar block in nucleo-cytoplasmic shuttling

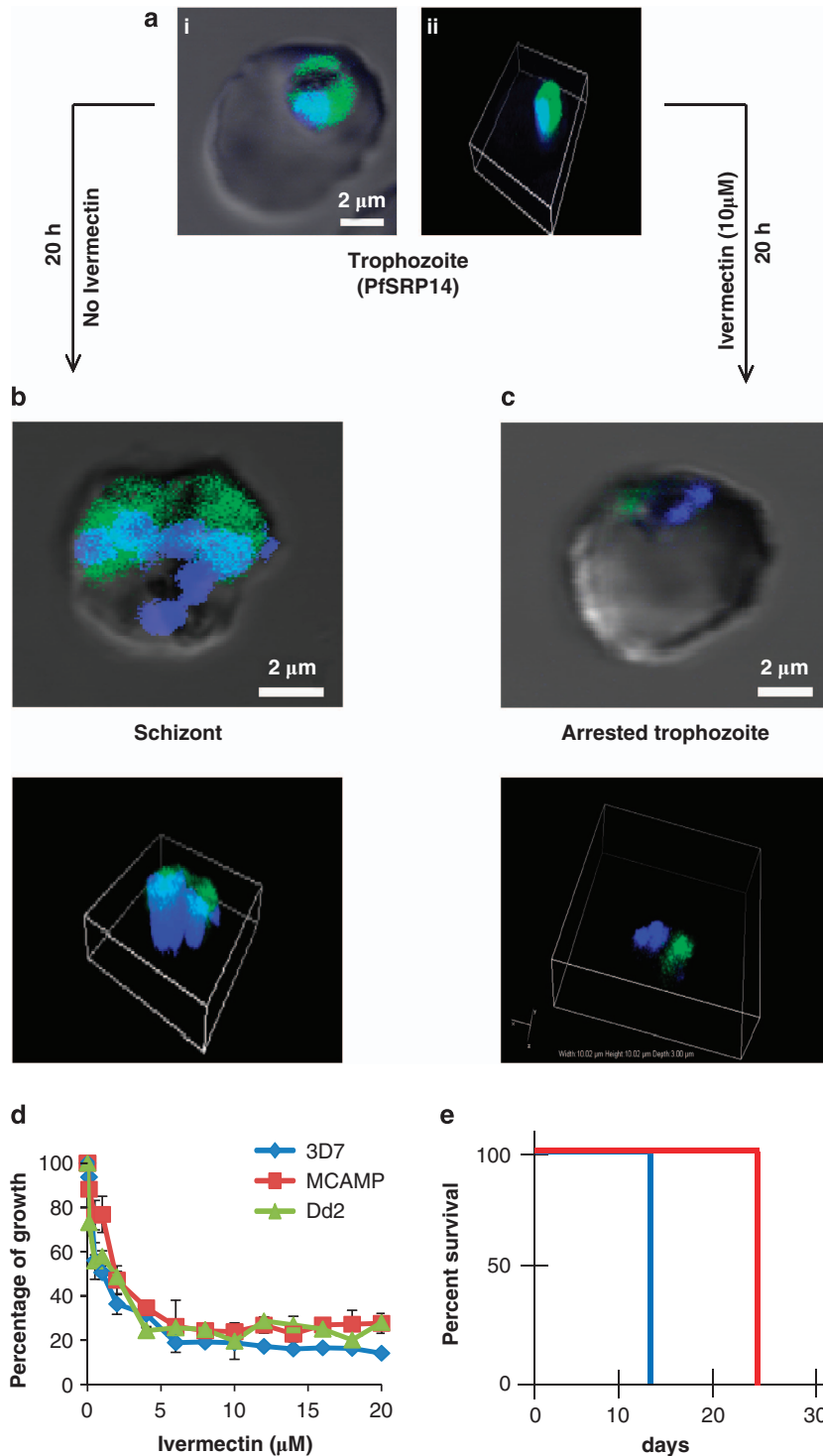


**Figure 5** Effect of aflatoxin B<sub>1</sub>, leptomycin B and ivermectin, on nucleo-cytoplasmic shuttling of GFP fused PfSRP9. Synchronised ring stage infected erythrocytes at 2% initial parasitemia were treated with these inhibitors and imaged at different time intervals. (a) upper panel, DMSO treated control parasite after 24 h of treatment, lower panel, AflatoxinB<sub>1</sub> (AFB<sub>1</sub>, 10  $\mu$ M) treated parasite at 24 h after treatment. (b) LeptomycinB (LMB, 25 ng/ml) treated parasite at 24 h after treatment and (c) parasite after 24 h of ivermectin (IVM) treatment at 10  $\mu$ M and 25  $\mu$ M, respectively



was observed for GFP fused PfSRP14 transgenic parasites (Figures 6b and c and supplementary videos 1 and 2). Subsequently, the effect of ivermectin on parasite growth was assessed by the SYBR green based assay on different

*P. falciparum* drug resistant strains; chloroquine (CQ)/mefloquine resistant (DD2 and MCamp) along with a chloroquine sensitive strain *P. falciparum* 3D7. Ivermectin exhibited anti-plasmodial activity against all the three



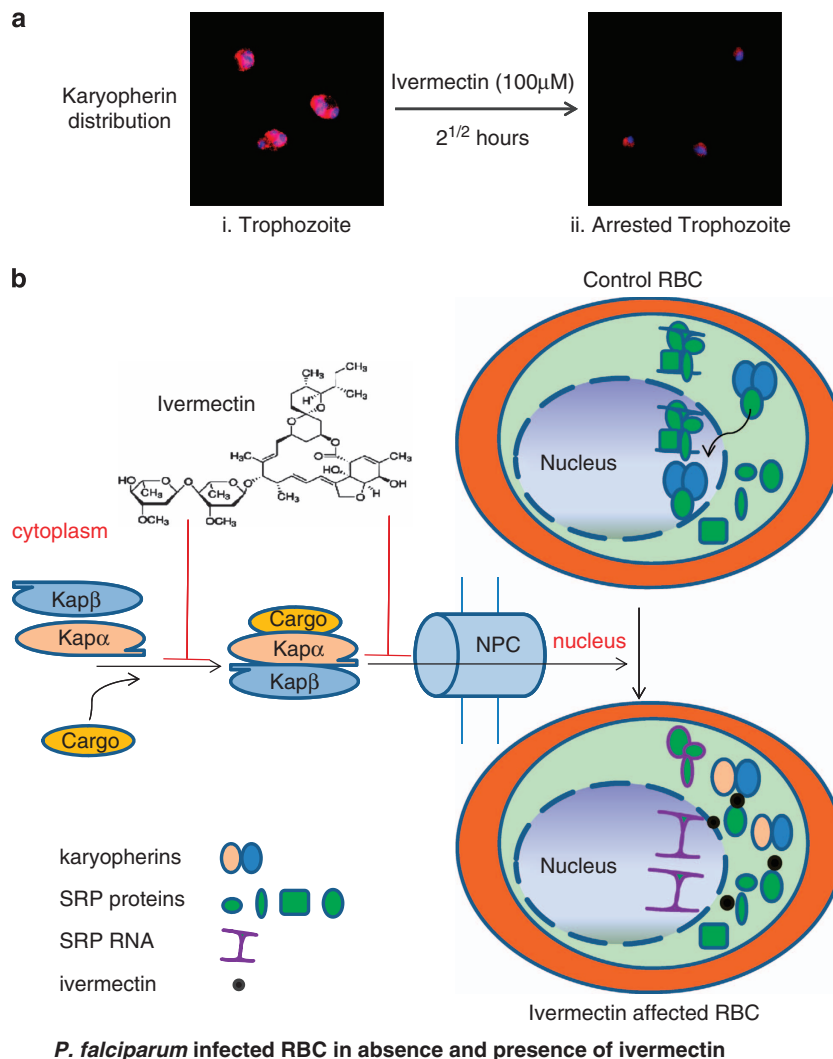
**Figure 6** Effect of Ivermectin on localisation of GFP fused PfSRP14 and growth of parasite. (a) i and ii show three dimensional reconstruction of confocal Z-stack merged images of GFP fused control trophozoite with DAPI (blue) as nuclear marker merged with DIC. (b) and (c), show the three dimensional reconstruction of confocal Z-stack merged images of GFP fused PfSRP14 with DAPI as nuclear marker merged with DIC in control and Ivermectin (10 µM) treated parasite at 24 h after treatment. (d) dose dependent effect of ivermectin on growth of *P. falciparum* 3D7, MCamp and Dd2 by SYBR Green assay (e) *In-vivo* effect of ivermectin against *P. berghei* in mice



strains Dd2, MCamp and 3D7 in a dose dependent manner with IC<sub>50</sub> values of 2.85, 1.92 and 1.56  $\mu$ M respectively (Figure 6d). We further tested the anti-malarial activity of ivermectin *in vivo* with *P. berghei* infected mice by administering a 0.5 mg/kg dose of ivermectin using a modified Thompson test (3 consecutive days of dosing). As seen in Figure 6e, ivermectin alone suppressed day 4 parasitemias by ~40% compared to placebo-treated controls. All four mice administered with ivermectin survived up to 23 days, while the control mice died on day 13 (Supplementary Figure S5c). Together these results suggest that ivermectin can be considered for the development as an antimalarial, which can be used in combination with other drugs.

Ivermectin is a known inhibitor of importin  $\alpha/\beta$  mediated nuclear transport.<sup>24</sup> *P. falciparum* has only two important

nuclear import factors; karyopherins  $\alpha$  and  $\beta$  in its genome, while the human genome encodes numerous importins. We next analysed the sub-cellular distribution of karyopherin  $\beta$  in ivermectin treated asexual blood stages of *P. falciparum* using anti-Pfkaryopherin  $\beta$  antibody.<sup>25</sup> Anti-Pfkaryopherin  $\beta$  recognized the parasite specific karyopherin  $\beta$  in untreated parasites and staining was mainly seen in and around the nucleus (Figure 7a, i). Intriguingly, parasites treated with ivermectin for 2<sup>1/2</sup> hours at 100  $\mu$ M concentration or with 5  $\mu$ M concentration for 20 h showed restricted staining in the cytoplasm (Figure 7a, ii and supplementary Figures S6a and b). Faint or no staining for PfSRP9 protein was observed at 20 h post exposure to 25  $\mu$ M of ivermectin, mainly because almost all the parasites were dead after this treatment (Supplementary Figure S6c). Together these results suggest



**Figure 7** Effect of ivermectin on localisation of karyopherin  $\beta$ , proposed mechanism of cargo import by karyopherin  $\alpha$ -karyopherin  $\beta$  pathway and possible mode of action of ivermectin. (a, i), control parasite with karyopherin  $\beta$  (red) and DAPI (blue) and ii, ivermectin (100  $\mu$ M) treated parasites after 2<sup>1/2</sup> h. (b) Karyopherin  $\alpha$  and  $\beta$  form a dimer and attach with the cargo and pass through the nuclear pore complex into nucleus where the cargo detaches itself from the karyopherins which are recycled into cytoplasm via RanGTP. SRP subunits join SRPRNA in the nucleus and the whole conglomerate is transported back to the cytoplasm where it becomes functional. Ivermectin blocks the parasite growth by inhibiting nucleo-cytoplasmic shuttling of SRPs essential for parasite survival either by affecting at the stage of karyopherin dimerisation or at the level of SRP attachment with Karyopherin dimer which inturn hampers the entry of SRP to its site of RNA assembly in the nucleus. Cell death occurs because of piling up of proteins in the cytoplasm

that ivermectin inhibits malaria parasite development by blocking nucleo-cytoplasmic shuttling of PfSRP components.

## Discussion

Recognition of the signal sequence in the ribosome nascent-chain (RNC) complex by SRP is the first step in protein secretion.<sup>26</sup> SRP couples the synthesis of membrane/secretory proteins and their transport across the ER membrane in eukaryotes<sup>27,28</sup> or across the bacterial plasma membrane<sup>8</sup> and chloroplast thylakoid membranes.<sup>29</sup> Considerable variations have been observed in the constituents of SRPs in different organisms.<sup>10</sup> Among the protozoan parasites, *T. brucei* and *Leptomonas collosoma* possess an unusual SRP that comprises two SRP RNAs; 7SL RNA and a tRNA like molecule and three SRP proteins; SRP19, SRP72 and SRP68, while the bacterial SRPs are of reduced complexity consisting of a small SRP RNA and a single protein SRP54, also referred as Ffh.<sup>10</sup> In the present study, *in silico* analysis revealed that the *P. falciparum* SRP is more similar to the mammalian SRP as it consists of six polypeptides; PfSRP72, PfSRP68, PfSRP54, PfSRP19, PfSRP14 and PfSRP9 and a single 303 nucleotide long PfSRP RNA. In comparison to the previous *in silico* studies, in the present study two additional PfSRP polypeptides; PfSRP68 and PfSRP72 were identified in the *P. falciparum* genome. It is important to mention here that the other members of the Alveolata to which *P. falciparum* belongs such as *Theileria*, *Eimeria* as well as members of the Euglenozoa such as *Trypanosoma* and *Leishmania*, lack one or two of the six SRP polypeptides. For example SRP9/14 has not been identified in *L. major*, *T. cruzi*, *Theileria annulata*, and *Giardia lamblia*.<sup>10,11</sup> A number of structural and protein–RNA interactions studies for *E. coli* and mammalian SRPs have provided insights into the assembly of SRPs and also delineated the interaction motifs (residues) between SRP54 and SRP RNA.<sup>30–32</sup> However, our understanding of SRPs in lower eukaryotes has been limited. Here we identified and report some of the essential features of *P. falciparum* SRP. The results demonstrate that all the components of mammalian SRP are conserved in the *P. falciparum* genome. We further show that *Plasmodium* SRP assembly process appears to be similar to that of human SRP assembly. The binding of PfSRP19 to PfSRP RNA most likely induces conformational changes in PfSRP RNA that subsequently binds to other components; such as PfSRP54, PfSRP14 and PfSRP9. Additionally, we demonstrate that during PfSRP assembly various PfSRP proteins also interact with each other, presumably to stabilize the PfSRP.

The sub-cellular localization studies performed for PfSRP proteins showed a clear nucleolar as well as cytoplasmic distribution for PfSRP9, PfSRP14 and PfSRP19 polypeptides at asexual blood stages by anti-PfSRPs antibodies and by GFP fluorescence analysis in transgenic lines. This was further confirmed by co-localization experiments with the nucleolar and ER markers. Importantly, PfSRP54 also showed nucleoplasmic distribution, although its level in the nucleus was low in comparison to other PfSRP polypeptides. Significantly, we could also observe PfSRP54 co-localization with PfNOP1, a nucleolar marker especially in earlier stages

of development. It has been proposed that nucleolus is the site of assembly and/or interaction between the families of mammalian ribonucleoproteins involved in protein synthesis, in addition to ribosomes themselves.<sup>33–35</sup> However, in mammalian cells, SRP54 does not seem to have a nucleolar phase.<sup>12</sup> Based on these findings, it has been suggested that in mammalian cells, SRP is partially assembled in the nucleus and it is piggybacked with ribosomal units for export from the nucleus.<sup>36</sup> In the cytoplasm, the partially assembled SRP is joined by SRP54 that recognizes the nascent signal sequence.<sup>33</sup> Based on the data for the localization of PfSRP proteins and mammalian SRP proteins, it seems more likely that in the case of PfSRP, complete assembly may occur in nucleus and this assembled complex is then transported to cytoplasm to perform ER targeting.

The movement of proteins between the nucleus and cytoplasm is an essential process and has been shown to be critical for the disease states such as parasitic diseases, viral diseases and oncogenesis. Although seven importin  $\alpha$ s and >20 importin  $\beta$ s have been described in human along with a wide range of NLS/NES sequences that are required for this transport, functional role(s) of each transporter in specific protein shuttling across nuclear membrane is not still understood.<sup>23</sup> Currently, the exportin/CRM1 inhibitor, Leptomycin B and importin  $\alpha/\beta$  inhibitor, ivermectin are the only accepted inhibitors being used to inhibit nuclear transport in mammalian cells. We used these inhibitors along with aflatoxin B<sub>1</sub> to study their effect on PfSRP polypeptides and their nucleo-cytoplasmic shuttling. Importantly, neither Leptomycin B, nor aflatoxin B<sub>1</sub> affected the localization and distribution of PfSRP-GFP polypeptides and these treated parasites grew normally in *in vitro* culture. In comparison, ivermectin, a specific inhibitor of importin  $\alpha/\beta$  blocked the nucleo-cytoplasmic shuttling of SRP polypeptides; PfSRP9-GFP and PfSRP14-GFP chimera proteins were mainly seen in the parasite cytoplasm in the ivermectin treated parasites. Ivermectin showed a potent inhibition in the growth of *P. falciparum* 3D7 as well as other drug resistant strains, Mcamp and DD2. Ivermectin treated *P. falciparum* parasites also showed a dramatic change in the level as well as the distribution of karyophorin  $\beta$ . Limited *in vivo* studies in a *P. berghei* mice model further demonstrated the antimalarial effect of ivermectin.

Ivermectin has been shown to inhibit replication of HIV-1 and Dengue virus by blocking the shuttling of nuclear proteins mediated by importin  $\alpha/\beta$ . In nematodes, ivermectin is known to bind and activate chloride ion channels,<sup>37</sup> a phenomenon which has been shown to be responsible for its anti-parasitic effect. Ivermectin also causes cell death in leukemia cells by inducing the chloride dependent membrane hyperpolarization. Based on these studies, it has been proposed that ivermectin affects the shuttling of importin  $\alpha/\beta$  by inducing the chloride dependent polarization of parasite nuclear membrane that in turn inhibits the nuclear import of SRP polypeptides. Since karyopherin  $\alpha/\beta$  are the only known importins present in *P. falciparum*, it is likely that ivermectin treatment is arresting parasite growth by blocking the shuttling of SRP polypeptides and some other nuclear proteins (Figure 7b). Ivermectin has recently been shown to inhibit the sporogony of *P. falciparum* in *Anopheles gambiae*.<sup>38</sup> Although a detailed *in vivo* analysis will be required for the use of ivermectin as an antimalarial

agent, however given the prior use of ivermectin and its safety record in humans and animals, it can be considered in combination therapy with other antimalarials. In conclusion, the results presented here provide insights into the malaria parasite PfSRP and show that ivermectin an anti-parasitic has the potential to treat multi-drug resistant malaria.

## Materials and Methods

**Generation of PfSRP-GFP lines.** To generate *P. falciparum*'s SRPs-GFP lines, genes corresponding to PfSRP54, -19, -14, -9 were PCR amplified and cloned into pSSPF2. The plasmids were transfected into *P. falciparum* 3D7 line and transfected cells were selected as described in detail in *SI text*.

## Analysis of protein–RNA and protein–protein interactions.

*In-vitro* interactions between PfSRPs and PfSRP RNA were performed using well established RNA binding assay as described in *SI text*. Protein–protein interactions between four PfSRP components were performed using bacterial two hybrid system and ELISA as described in *SI text*.

**Effect of inhibitors on PfSRP-GFP transgenic lines.** *P. falciparum* culture was synchronised by two consecutive sorbitol treatments at an interval of four hours. The parasite culture was incubated with different concentrations of leptomycinB, aflatoxin B1 and ivermectin as described in *SI text*.

**Growth inhibition assay on *P. falciparum* 3D7, Dd2 and mCAMP strains.** Parasite culture with an initial parasitemia of 0.8% was treated with different doses of leptomycin B, aflatoxin B1 and ivermectin in 96 well plate and new ring formation was monitored as described in *SI text*.

## Conflict of Interest

The authors declare no conflict of interest.

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