

Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles

Neta Regev-Rudzki^{1,2,*}, Danny W Wilson^{1,2,*}, Teresa G Carvalho^{1,2,6}, Xavier Sisquella^{1,2}, Bradley M. Coleman^{3,4}, Melanie Rug^{1,2,7}, Dejan Bursac^{1,2}, Fiona Angrisano^{1,2}, Michelle Gee⁵, Andrew F. Hill^{3,4}, Jake Baum^{1,2} and Alan F Cowman^{1,2}

¹The Walter and Eliza Hall Institute of Medical Research, Parkville 3052, Australia

²Department of Medical Biology, ³Department of Biochemistry and Molecular Biology,

⁴Bio21 Molecular Science and Biotechnology Institute,

⁵School of Chemistry, The University of Melbourne, Parkville, Victoria, Australia

* These authors contributed equally.

Correspondence to:

Alan F. Cowman

The Walter and Eliza Hall Institute

1G Royal Parade

Melbourne, 3050

Australia

Telephone: 61-3-9345446

Facsimile: 61-3-93470852

Email: cowman@wehi.edu.au

⁶ Current address: Department of Microbiology, Monash University, Clayton 3168,

Australia

⁷ Current address: Centre for Advanced Microscopy, The Australian National University,

Canberra 0200, Australia

SUMMARY

Cell-cell communication is an important mechanism for information exchange promoting cell survival to control features such as population density and differentiation. We determined that *Plasmodium falciparum*-infected red blood cells directly communicate between parasites within a population using exosome-like vesicles that are capable of delivering genes. Importantly, communication via exosome-like vesicles promotes differentiation to sexual forms at a rate suggesting signaling is involved. We have further identified a *P. falciparum* protein, PfPTP2, which plays a key role in efficient communication. This study reveals a previously unknown pathway of *P. falciparum* biology critical for survival in the host and transmission to mosquitoes. This identifies a new pathway for development of novel agents to block parasite transmission from the human host to the mosquito.

Keywords: malaria, cell-cell communication, gametocytes, sexual differentiation.

INTRODUCTION

Cell-cell communication and cooperative motility are well known in multicellular eukaryotes and include tissue morphogenesis, wound healing and tumour metastases. Direct communication between mammalian cells occurs either by transfer of information through microvesicles or physical connection through nanotubes (Belting and Wittrup, 2008; Gerdes and Carvalho, 2008). Extracellular vesicles (EVs) are small cellular particles of 30-400 nm and their release is highly conserved in biology (reviewed in (Delabranche *et al.*, 2012)). EVs are divided into microparticles (MP), microvesicles (MV) and exosomes based on size and cell compartment from which they originate. MPs (100-400 nm) are released by vesiculation of plasma membranes whereas exosomes (30-150 nm) originate from multivesicular bodies (MVB) derived from late endosomes (Johnstone *et al.*, 1987). Exosomes and MPs are involved in transfer of biologically active molecules to induce phenotypic changes (Belting and Wittrup, 2008; Coleman *et al.*, 2012; Ratajczak *et al.*, 2006). In malaria, MPs are released from infected red blood cells (RBC) and activate endothelium at the blood brain barrier, exacerbating inflammation (Combes *et al.*, 2005; Nantakomol *et al.*, 2011).

Whilst cell-cell communication and social behaviour is established in many eukaryotes and prokaryotes (Bassler and Losick, 2006; Dubey and Ben-Yehuda, 2011; Record *et al.*, 2011) there is less known for parasitic protozoa (Lopez *et al.*, 2011; Rupp *et al.*, 2011). Protozoan parasites are responsible for major diseases including malaria, caused by the genus *Plasmodium*. *P. falciparum* and *P. vivax* are responsible for most clinical cases of malaria in humans. These vector-borne parasites cycle between mosquitoes to humans and in both contexts are faced with an unstable and hostile environment. To ensure survival and transmission, the malaria parasite

must infect and survive in the human host and differentiate into sexual forms (gametocytes), which are competent for transmission to mosquitoes. The molecular mechanisms for commitment to gametocytogenesis, a ‘once in a life cycle decision’, are obscure in *Plasmodium* biology (reviewed in (Alano, 2007)).

In the protozoan parasite *Trypanosoma brucei*, responsible for sleeping sickness, a density-sensing mechanism activates differentiation of proliferative slender cells to stumpy forms through release of stumpy induction factor (STI) (MacGregor *et al.*, 2011; Reuner *et al.*, 1997; Vassella *et al.*, 1997). Stumpy forms are committed to cell cycle arrest in the host, limiting population density, and are competent for transmission to the tsetse fly. This quorum sensing-like mechanism provides a means for *T. brucei* to enhance vector transmission and prolong survival in the host (MacGregor *et al.*, 2011).

It would be a major advantage for *Plasmodium* parasites, such as *P. falciparum*, to communicate during blood stage infection to enable populations to react to changing conditions in the host. To date no study has provided direct evidence for its existence. Here we demonstrate cell-cell communication between *P. falciparum* parasites through exosome-like vesicles that promotes differentiation to sexual forms.

RESULTS

Communication between *P. falciparum* cells mediate DNA-dependent transfer of drug resistance and fluorescence

To determine if *P. falciparum*-infected RBCs communicate and transfer information we used strains expressing different drug resistance cassettes and fluorescent proteins as markers. The parasite line 3D7^{dhfr^{GFP}}, has an episomal (e) plasmid, expressing *human dihydrofolate reductase* (*dhfr*) conferring resistance to WR99210 (WR), and green fluorescent protein (GFP) (Boddey *et al.*, 2010). A second line CS2eBsd^{GFP},

contains an episomal (e) plasmid expressing blasticidin deaminase (Bsd), conferring resistance to blasticidin-S (Bs), and GFP (Ataide *et al.*, 2010). Parasite lines were co-cultured in Bs+WR and after initial cell death, expected due to both drugs, parasites grew to high parasitaemia within 5 days (Fig. 1 A and B). In contrast, when CS2eBsd^{GFP} or 3D7edhfr^{GFP} (parental lines) were cultured alone in both drugs no parasites survived.

We tested other *P. falciparum* lines to determine if they transferred drug resistance between cells. The WR resistant CS2idhfr⁹²⁰ has an integrated (i) copy of *hdhfr* and was co-cultured with Bs resistant CS2eBsd^{GFP} in Bs+WR with ring stage parasites observed after 5 days (Fig. 1 C). To test if this was due to release of plasmid into supernatants or uptake by parasitised RBCs we added CS2idhfr⁹²⁰ to RBCs electroporated with a plasmid conferring Bs resistance (Fig. 1 C). Typically for this method of *P. falciparum* transfection parasites are observed at 21 days (Fidock and Wellems, 1997). However, when plasmid loaded RBCs were incubated with CS2idhfr⁹²⁰ no ring stage parasites were obtained after 5 days on Bs. Additionally, we added plasmid encoding a *bsd* gene to CS2idhfr⁹²⁰ parasites and again no ring stage parasites were detected after 5 days on Bs. Therefore *P. falciparum* lines can rescue parasite growth, under drug selection, when co-cultured and this was not through uptake of plasmid DNA released during normal growth.

If drug resistance genes were transferred to cells in the population *via* cell-cell communication then fluorescent proteins encoded on the plasmid should be expressed. 3D7idhfr^{mCh} expresses mCherry in the nucleus (Volz *et al.*, 2010) whilst CS2eBsd^{GFP} expresses GFP in the cytoplasm (Fig. 1 D) (Ataide *et al.*, 2010). We detected dual coloured (red nucleus and green cytoplasm) parasites following 5 days of co-culture for CS2eBsd^{GFP}+3D7idhfr^{mCh} with Bs+WR (Fig. 1 D). To test if transfer

of GFP or mCherry expression between parasites resulted from plasmid transfer we used fluorescence *in-situ* hybridization (FISH) to detect *bsd* (CS2eBsd^{GFP}) and *hdhfr* (3D7edhfr^{GFP}) genes in co-cultured CS2eBsd^{GFP}+3D7edhfr^{GFP} parasites with Bs+WR (Fig. 1 E). In the co-culture experiments analysed by FISH 100% of the parasites were positive for both *hdhfr* and *bsd* genes confirming transfer of an episomal plasmid. In contrast, the parental parasites were positive for only the endogenous drug resistance marker.

DNA-dependent transfer was confirmed by PCR of 3D7idhfr^{mCh}+CS2eBsd^{GFP} in Bs+WR (Fig. 1 F). The genes *hdhfr* and *mcherry* were present in 3D7idhfr^{mCh} and conversely *bsd* and *gfp* were detected in CS2eBsd^{GFP} as expected for parental lines. When 3D7idhfr^{mCh} and CS2eBsd^{GFP} were co-cultured in Bs+WR, *hdhfr*, *mcherry*, *bsd* and *gfp* genes were detected. This was not due to remnant gDNA from dead or dying cells as no genes were detected by PCR for 3D7idhfr^{mCh} and CS2eBsd^{GFP} parental lines cultured separately in Bs+WR (Fig. 1 F). Together these data show *P. falciparum* parasites can transfer information between cells in a population.

Parasite communication is mediated by factors released by *P. falciparum*-infected RBCs

To determine if communication between *P. falciparum*-infected RBCs required cell contact we used transwells to physically separate parasites in culture. We established *P. falciparum*-parasites could not pass through 400 nm pore size transwell membranes. We separated 3D7edhfr^{GFP} and CS2eBsd^{GFP} in transwells with Bs+WR and assessed ring stage parasitaemia after 5 days (Fig. 2 A). 3D7edhfr^{GFP} survived in either the insert or bottom of the transwells only if CS2eBsd^{GFP} was present in the opposite chamber. Interestingly, when placing the two parasite lines in different

compartments of the transwell directionality of transfer was towards 3D7edhfr^{GFP} and not CS2eBsd^{GFP} implying it was the drug selection cassette (*bsd*) and not the episomal plasmid *per se* determining direction of transfer. The predominance of episomal *bsd* plasmid transfer was likely because Bs inhibits ring and early trophozoite stages (Fig. S1) whereas WR inhibits later in the lifecycle (Dieckmann and Jung, 1986). In these experiments we add both drugs at ring stages and we show below that ring stage is critical for cell-cell communication.

Experiments with 3D7idhfr^{mCh} and CS2eBsd^{GFP} gave similar results with Bs drug resistance transferred to 3D7idhfr^{mCh} (Fig. 2 B). In related experiments plasmid transfer from CS2eBsd^{GFP} to a second integrated line, CS2idhfr⁹²⁰, was confirmed by PCR amplification of *hdhfr* and *gfp* genes in co-culture transwells containing CS2idhfr⁹²⁰ after removal of CS2eBsd^{GFP} in inserts (Fig. 2 C). Therefore factors are released into supernatants that carry a DNA plasmid through a 400 nm pore to communicate with *P. falciparum*-infected RBCs. This demonstrates communication between *P. falciparum* parasites does not require direct cell-cell contact and occurs over long distances.

We next exploited transwells to study whether DNA-carrier factors present in co-cultured media were stable and could ‘rescue’ a drug resistance phenotype. Increasing volumes of 3D7edhfr^{GFP}+CS2eBsd^{GFP} parasites were cultured in transwell inserts with Bs+WR (Fig. 2 D). After 24 hr of co-culture the insert was removed and naïve recipient 3D7edhfr^{GFP} added with Bs+WR to the parasite-free medium. Remarkably, the initial presence of 3D7edhfr^{GFP}+CS2eBsd^{GFP} in the insert rescued 3D7edhfr^{GFP} growth, consistent with transfer of Bs resistant plasmids. Moreover, there was an increase in rescue of 3D7edhfr^{GFP} associated with increasing volumes of the initial co-culture (Fig. 2 D). Multiple experiments comparing co-cultured versus

single lines gave similar levels of rescue suggesting efficiency was the same (Fig. S2). These data demonstrate stable factors are secreted into supernatants by *P. falciparum*-infected RBCs that mediate transfer of drug resistance to other parasites.

To determine if the dose response measured for communication activity was due to continuous release of factor(s) between *P. falciparum* cells we incubated ring stage donor, CS2eBsd^{GFP}, in the insert and recipient 3D7edhfr^{GFP} in the bottom with Bs+WR (Fig. 2 E). The inserts were removed immediately or between 1 and 24 hr after mixing. Increasing exposure of 3D7edhfr^{GFP} to CS2eBsd^{GFP} in inserts resulted in increased survival consistent with greater transfer of Bs resistance. Our results demonstrate rapid and efficient plasmid transfer, even after 1 hr, with maximal rescue of the 3D7edhfr^{GFP} line occurring within 10 hr of co-culture of ring stages. This suggests *P. falciparum* employs an efficient mechanism to communicate and transfer factors harbouring cellular information over long distances without direct cell-cell contact.

Communication occurs at ring stages and is sensitive to actin filament and microtubule inhibitors

We examined timing of communication and plasmid transfer within the blood stage asexual cycle by co-culture of either ring or trophozoites of 3D7idhfr^{mCh}+CS2eBsd^{GFP} in Bs+WR (Fig. 3 A). Ring stage co-culture showed efficient plasmid transfer while parasite survival for trophozoite co-cultures was 10-fold less. This suggests that efficient communication and plasmid transfer occurs mainly in ring stages of the asexual life cycle.

Since drug selection of *P. falciparum* parasites cause stress and death we addressed if this affected cell-cell communication. Using transwells, we co-cultured 3D7edhfr^{GFP}+CS2eBsd^{GFP} parasites in inserts with or without drugs for 24 hr (Fig. 3

B). The insert was removed and naïve 3D7edhfr^{GFP} added to cell-free medium containing Bs+WR. Growth of 3D7edhfr^{GFP} in wells where no drugs were initially added, in the first 24 hr, was greatly reduced compared to wells where both Bs+WR were present through experiments. While suggestive of more efficient transfer of Bs resistance during drug stress, in the absence of drugs 3D7edhfr^{GFP}+CS2eBsd^{GFP} parasites still transferred Bs resistance to 3D7edhfr^{GFP}. Our study shows that intercellular communication between *P. falciparum*-infected RBCs occurs under normal conditions; however, signalling between cells under stress conditions, such as when faced with antimalarials in the host, is a more active process. The importance of ring stages and stress in cell-cell communication suggests the directionality of episomal Bs plasmid transfer observed in Figures 2A and B may be due to Bs acting against ring stages and causing stress, while WR acts later and does not cause stress at the critical ring stage for cell-cell communication.

As communication and plasmid transfer occurs efficiently at ring stages without direct contact between cells we hypothesised EVs were providing a vectorised and efficient mode of export and signalling. Since actin and microtubules have a role in secretion of vesicles, we tested sub-lethal concentrations of inhibitors of these processes for a role in *P. falciparum* communication (Dieckmann-Schuppert and Franklin, 1989; Shaw et al., 2000). Cytochalasin D (CytoD) and oryzalin (ORY) were potent inhibitors of plasmid transfer between 3D7edhfr^{GFP} and CS2eBsd^{GFP} (Fig. 3 C). Significant inhibition (75%) was also observed for swinholide (SWIN). Both CytoD and oryzalin inhibition showed a dose-dependent response at sub-lethal levels for co-cultured Bs+WR treated parasites (Fig. 3 D and E). CytoD and oryzalin are inhibitors of actin polymerisation and microtubule depolymerisation respectively suggesting these functions are required for cell-cell communication. In addition, we found

heparin blocked plasmid transfer in *P. falciparum* consistent with studies showing highly charged heparin suppresses microvesiculation (Sustar *et al.*, 2009).

Exosome-like vesicles are released into the culture supernatant

To address the size of mediators of *P. falciparum* cell-cell communication we cultured CS2eBsd^{GFP} enclosed in dialysis tubing (excludes <100 kDa) with 3D7edhfr^{GFP} recipient in the outside compartment (Fig. 4 A). Whilst in control experiments 3D7edhfr^{GFP} grew normally when CS2eBsd^{GFP} was separated by dialysis tubing, it did not grow in the presence of Bs+WR indicating the mediator(s) responsible for communication was >100 kDa.

We next used Atomic Force Microscopy (AFM) to determine if EVs were present in supernatants and if their concentration changed with differing treatments of *P. falciparum*. We visualised *P. falciparum*-infected RBCs by AFM and observed vesicles (~120 nm diameter) around parasites (Fig. 4 B, black arrows) as well as small protrusions on the RBC (Fig. 4 B, white arrows) that may be in the process of being released (Fig. 4 B). Whilst this was insufficient to conclude these are vesicles being actively released, it suggested this process may occur from *P. falciparum*-infected RBCs and we explored this in more detail.

To determine if EVs were present in supernatant released from *P. falciparum*-infected RBCs, we incubated CS2eBsd^{GFP}+3D7edhfr^{GFP} with Bs+WR (Fig. 4 C, Mix++) or no drug (Fig. 4 C, Mix) in transwell inserts and harvested supernatants from the bottom for AFM. Remarkably, vesicles of a similar width were observed both in presence (123.1 ± 2.08 nm) and absence of drug (112.2 ± 17.7 nm) in cell-free supernatants of CS2eBsd^{GFP}+3D7edhfr^{GFP}. Vesicle height was similar in both cases (13.1 ± 0.9 and 12.6 ± 1.6 nm, respectively). As predicted, supernatant in absence of drugs showed vesicles are present at lower numbers (1.5 ± 0.4 vesicles in $100 \mu\text{m}^2$

area) compared to parasite mixtures in Bs+WR ($8.5 \pm 2.2/100 \mu\text{m}^2$) (Fig. 4 C). Some vesicles appeared to be wider than the low-pass of transwell filters (400 nm) likely because of aggregation and spreading on mica surfaces as well as AFM tip convolution (Fig. 4 D). Importantly, incubation of sub-lethal concentrations of communication inhibitor CytoD (Fig. 3 C and D) reduced the number of vesicles ($2/100 \mu\text{m}^2$) (width $89.73 \pm 3.04 \text{ nm}$, height $8.1 \pm 1.4 \text{ nm}$) (Fig. 4 C, Mix ++ CytoD). Vesicles were also observed in RBC supernatants (width $106.5 \pm 1.12 \text{ nm}$, height $7.3 \pm 0.6 \text{ nm}$) but the number was substantially less than that observed with *P. falciparum*-infected RBCs. Features of 10 to 100 nm width, with an average height of 5 nm, were observed in culture medium consistent with a background deposition of protein aggregates (Fig. 4 C, RBC and Media). The quantity of vesicles released from *P. falciparum*-infected RBCs correlates with frequency of plasmid transfer and this implicates them as mediators of intercellular communication. The size of these vesicles suggests they are analogous to mammalian exosomes, sharing some features such as release from viable cells (Bang and Thum, 2012; Record et al., 2011).

Exosome-like vesicles are responsible for cell-cell communication and plasmid transfer

In order to determine if exosome-like vesicles, identified in culture supernatants by AFM, were responsible for cell communication and plasmid transfer we attempted to purify them. A method employing Optiprep density gradient centrifugation was used to fractionate the culture medium (Coleman et al., 2012). Purification of vesicles using these gradients and analysis of plasmid transfer in fractions resolved a discrete peak of activity observed in fractions 4 and 5 consistent with buoyant vesicles being responsible (Fig. 5 A).

We used AFM as well as negative-staining and cryo-TEM to visualize

contents of Optiprep fractions. In fraction 4 and 5 a relatively homogeneous population of ~70 nm diameter spherical vesicles were observed (using AFM sizes were: width: 70.55 ± 3.92 , height: 12.37 ± 1.04) (Fig. 5 B and C). In contrast, vesicles of similar size were not present in fraction 3 and 6, although smaller aggregates of ~14 nm were observed by TEM and AFM (using AFM sizes were: width: 14.13 ± 1.37 , height: 4.28 ± 0.87) as a rough background. The lack of plasmid transfer activity in fraction 3 was consistent with the smaller aggregates having no role in cell-cell communication. However, the predominance of the ~70 nm vesicles banding with cell-cell communication activity suggests they are likely responsible. These vesicle dimensions are comparable to those observed by AFM (~120 nm diameter) when spreading on the mica surface is taken into account. Together, these data provide strong evidence that exosome-like vesicles are responsible for cell-cell communication between *P. falciparum*-infected RBCs.

An exported *P. falciparum* protein is required for efficient communication

We hypothesised that *P. falciparum* proteins required for communication between parasite-infected RBCs would include those involved in trafficking to host cells. Previously, we performed a gene knockout screen and identified proteins, named PfEMP1 Trafficking Protein (PfPTP), required for trafficking of virulence protein *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Maier et al., 2008). PfEMP1 is trafficked to membranous structures called Maurer's clefts that bud from the parasitophorous vacuolar membrane, migrate to the underside of host cells then insert into the RBC membrane (Kriek et al., 2003; Papakrivos et al., 2005). We showed parasites CS2idhfr^{ΔPTP1}, CS2idhfr^{ΔPTP2} (Fig. S3) and CS2idhfr^{ΔPTP3} do not transfer PfEMP1 to the parasite-infected RBC surface (Maier et al., 2008). These knockout parasites were tested to determine if they could receive a plasmid from donor

CS2eBsd^{GFP}. Transfer of resistance to Bs was efficient for CS2idhfr^{ΔPTP1} and CS2idhfr^{ΔPTP3}, however, it was greatly reduced for CS2idhfr^{ΔPTP2} (Fig. 6 A). There was no significant difference between CS2idhfr^{ΔPTP2} and CS2idhfr^{ΔPTP1} growth rates in the absence of Bs indicating that the loss of PfPTP2 reduces cell-cell communication in co-culture experiments. These experiments demonstrate PfEMP1 is not required for efficient communication and suggests PfPTP2 plays a role in communication and plasmid transfer.

To address potential roles of PfPTP2 in intercellular communication we determined its subcellular localisation using super-resolution microscopy. We used CS2idhfr^{PTP2/HA}-infected RBCs (Fig. S3) and identified large numbers of structures in host cell cytoplasm some of which appeared to be budding from Maurer's clefts (labelled using antibodies to skeleton binding protein1, SBP1) (Fig. 6 B) (Maier *et al.*, 2007). PfPTP2 was further localised to membranous structures budding from Maurer's clefts by immuno-electron microscopy, suggesting that PfPTP2 labelled material in host cell cytoplasm were derived from these membranous organelles (Fig. 6 C panels a and inset, b, c). Solubility of PfPTP2 was consistent with its association on the outer membrane of Maurer's clefts and vesicles, either through binding to lipid or a protein(s) in the membrane (Fig. S4). These results suggest PfPTP2 functions in budding of vesicles from Maurer's clefts.

Further, if these cytoplasmic vesicles were the source of extracellular vesicles then CS2idhfr^{ΔPTP2} parasites, which lack the function of PfPTP2, would have greatly reduced quantities in the extracellular space. To examine this AFM was used to quantitate vesicles in supernatants of CS2idhfr^{ΔPTP2} compared to CS2idhfr^{ΔPTP1} (Fig. 6 D). Indeed, CS2idhfr^{ΔPTP2} (0.2 ± 0.1 vesicles/100 μm^2 , width 74.8 ± 8.3) released 16-fold less vesicles into the supernatant than CS2idhfr^{ΔPTP1} (3.2 ± 1.1 vesicles/100 μm^2 ,

width 76.6 ± 1.62) suggesting these particles are derived from the intracellular PfPTP2 coated vesicles (Fig. 6 D). Moreover, this result was consistent with the significantly impaired ability of CS2idhfr^{ΔPTP2} for cell-cell communication. These data strongly suggest PfPTP2 functions in budding of vesicles from Maurer's clefts and release of exosome-like vesicles into supernatant, implicating *P. falciparum*'s molecular machinery in communication.

Communication between *P. falciparum* parasites increases sexual differentiation

Whilst it was clear that co-cultured *P. falciparum* lines efficiently transfer DNA, we made the interesting observation that blood stage parasites disappeared over 2 weeks and were replaced by increased levels of gametocytes (sexual forms). To explore if communication between *P. falciparum* cells promotes sexual differentiation we determined if gametocytes originating from co-cultured 3D7idhfr^{mCh} (mCherry) and 3D7iBsd^{CK1-GFP} (GFP) on Bs+WR expressed both fluorescent proteins (Fig. 7 A). Live fluorescence microscopy of different stages of developing gametocytes showed cytoplasmic GFP and nuclear mCherry confirming transfer of plasmids. The plasmids in these parasites are integrated via single recombination and consequently can frequently loop out to reform episomal plasmids. Thus it is likely these have been transferred as plasmids rather than directly from a chromosomal location.

Next we compared the quantity of gametocytes produced by 3D7edhfr^{GFP} and CS2eBsd^{GFP} when co-cultured in Bs+WR. Gametocytes were quantified for 3D7edhfr^{GFP} and CS2eBsd^{GFP} cultured alone with few gametocytes observed (Fig. 7 B). In contrast, 17-fold more gametocytes were seen for 3D7edhfr^{GFP}+CS2eBsd^{GFP} co-cultures (Fig. 7 B). Similar results were observed for combinations of 3D7idhfr^{mCh} and 3D7iBsd^{CK1-GFP} parasites (Fig. 7 C). Further, in a complementary approach to fully quantify gametocytogenesis, we used FACS analysis of Bs+WR treated

3D7edhfr^{GFP} and CS2eBsd^{GFP} parental line and co-cultures with N-acetyl glucosamine (NAG) depletion of asexual, but not gametocyte, forms (Gupta *et al.*, 1985) (Fig. 7 D, E). Again 3D7edhfr^{GFP} and CS2eBsd^{GFP}, when cultured alone, produced low levels of gametocytes whereas mixtures in drugs efficiently produced high levels. The 3D7idhfr/Bsd^{Δ175/181} line, which has both *hdhfr* and *bsd* inserted into the genome (Fig. 7 D, F) (Lopaticki *et al.*, 2011), was used as control and showed gametocyte production, while clearly evident, was much less than co-cultured parasites. The level of conversion of Bs+WR selected cells from surviving blood stage parasites to gametocytes in mixed cultures was very efficient and these experiments suggest most, if not all, of the population that received a plasmid underwent sexual differentiation to gametocytes. These findings demonstrate cell-cell communication between *P. falciparum* parasites allows parasite survival and increased differentiation of gametocytes for disease transmission.

DISCUSSION

Cell-cell communication and social behaviour of cells within a population has become a common feature in organisms ranging from higher eukaryotes to single cell eukaryotes and bacteria (Ratajczak *et al.*, 2006; Belting and Wittrup, 2008). This social and cooperative behaviour plays an important role in many different processes ranging from cell differentiation to development of bacterial and single cell eukaryotic ecosystems to enhance survival (Gerdes and Carvalho, 2008; Lopez *et al.*, 2011; Marzo *et al.*, 2012). Protozoan parasites such as *T. brucei* have a population sensing mechanism important for transmission to the insect vector (MacGregor *et al.*, 2011; Reuner *et al.*, 1997; Vassella *et al.*, 1997). Here we show that *P. falciparum*-infected RBCs are capable of transferring DNA within the population via EVs, which we have termed exosome-like. Importantly, these exosome-like vesicles are shed from

P. falciparum-infected RBCs and allow parasites to transfer, receive and propagate information that is advantageous for population growth under stressed and non-stressed conditions. Further, cell-cell communication facilitates differentiation and activation of parasites competent for transmission to mosquito vectors. Increased production of exosome-like vesicles under conditions of stress, such as drug pressure, would be highly advantageous for parasite survival in providing a means to react to environmental conditions. In other words, *P. falciparum* promotes differentiation of sexual forms and escape to the vector in response to conditions in the host less conducive for survival.

The EVs released from *P. falciparum*-infected RBCs are termed exosome-like since they are similar in size to mammalian exosomes and share common features such as being released from viable cells (Bang and Thum, 2012; Record *et al.*, 2011). It is not clear whether these vesicles are the same as MPs derived by vesiculation of RBC membranes involved in stimulation of pro-inflammatory responses (Couper *et al.*, 2010) but our finding that there are subpopulations of vesicles with different sizes suggests they may be functionally distinct. Optiprep gradients have been used previously to purify exosomes from human cells and provides a method for high resolution separation of vesicles (Coleman *et al.*, 2012). Using this methodology we have shown *P. falciparum* cell-cell communication activity was restricted to specific fractions. Visualization of the contents of fractions by AFM and electron microscopy showed they contained spherical vesicles of ~70 nm diameter. The ability to purify these vesicles provides the opportunity for further downstream analysis including proteomics, lipidomics, genomics and structural biology to further define functional characteristics of these exosome-like vesicles.

Identification of *P. falciparum* protein PfPTP2, which plays a role in mediating intercellular communication, suggests exosome-like vesicles may be derived from Maurer's clefts and not RBC membranes. The PfPTP2 coated particles in *P. falciparum*-infected RBCs appear to be vesicular structures, previously defined as electron dense vesicles (EDVs), and may be related to other particles called J-dots (Hanssen et al., 2010; Kulzer et al., 2010). Whilst function of EDVs is unknown, their size is consistent with that observed for PfPTP2 coated structures (Hanssen et al., 2010). The localisation of PfPTP2 coated vesicles in the process of budding from Maurer's clefts suggests they are formed from these large vesicular structures that play a role in protein sorting, targeting and packaging and as such have similarities to late endosomes. Whether the PfPTP2 coated vesicles are equivalent to MVBs and whether the exosome-like vesicles originate directly from them by secretion across the RBC plasma membrane remains to be determined. Disruption of PfPTP2 function decreases the number of extracellular exosome-like vesicles and this is consistent with PfPTP2 labelled vesicles having a pivotal role in genesis and transmission of exosome-like vesicles. However, it is also clear that PfPTP2 is important for receipt of the signal by the target cell.

The ability of *P. falciparum* to differentiate from blood stage asexual to sexual forms is essential for transmission to mosquito vectors (reviewed in (Alano, 2007)). How this process is activated and regulated is not known. Our study demonstrates that exosome-like vesicles released from *P. falciparum*-infected RBCs enable survival of drug treated parasites and lead to greatly increased numbers of gametocytes competent for transmission to the next host. Intriguingly, in *T. brucei* stumpy induction factor (STI) leads to increased differentiation to the transmission competent stumpy form at high parasite loads (MacGregor et al., 2011; Reuner et al., 1997;

Vassella *et al.*, 1997). The high levels of gametocytes achieved during cell-cell communication here suggest active signalling of gametocytogenesis. This is supported by demonstration that vesicles purified from culture supernatants act as a messenger to induce gametocytogenesis (Mantel *et al.*, 2013). The exact nature of any activation signal is unknown, however, exosomes in mammalian cells are key players in signalling between cells and are known to transfer mRNA, miRNA, lipid mediators and proteins (Bang and Thum, 2012; Record *et al.*, 2011).

In summary, we have demonstrated that cell-cell communication occurs between *P. falciparum*-infected RBCs and that this provides a mechanism to increase parasite survival in times of stress and promote differentiation to sexual forms. This is a key advantage for parasites in maintaining infection of the human host to maximise chances of transmission to the mosquito vector (MacGregor *et al.*, 2011). We further provide evidence that exosome-like vesicles are responsible for parasite communication. Moreover, we identified a key *P. falciparum* protein, which is for this pathway. It is likely that many other *P. falciparum* proteins are involved in the development and secretion of these vesicles in the donor cells as well as detecting the signal in the recipient. This process is potentially an excellent target for novel therapeutic approaches to block *P. falciparum* transmission to the vector and will be an important factor that requires addressing with respect to the spread of further parasite drug resistance.

EXPERIMENTAL PROCEDURES

Parasite lines

Parasite lines: CS2eBsd^{GFP} (Ataide *et al.*, 2010); 3D7edhfr^{GFP} (GFP fused to PfEMP3, PF3D7_0201900) (Boddey *et al.*, 2010); 3D7idhfr^{mCh} (mCherry fused to PF3D7_0919000) (Volz *et al.*, 2010); 3D7iBsd^{CK1-GFP} (GFP fused to CK1,

PF3D7_1136500) (D. Dorin-Semblat and C. Doerig, unpublished); CS2idhfr⁹²⁰ (knock-out of PFB0920w/PF3D7_0220100); CS2idhfr^{ΔPTP1}, CS2idhfr^{ΔPTP2} and CS2idhfr^{ΔPTP3} (knock-out of PFB0106c/PF3D7_0202200, PfPTP1; MAL7P1.172/PF3D7_0731100, PTP2 and PF14_0758/PF3D7_1478600, PTP3, respectively) (Maier et al., 2008); 3D7idhfr^{175/181} (3D7 line with knock-outs of EBA-175 and EBA-181) (Lopaticki et al., 2011). CS2idhfr^{PTP2/HA} (*Pfptp2* gene, MAL7P1.172/PF3D7_0731100, tagged with HA) (Fig. S3).

Parasite culture and co-culture experiments

P. falciparum parasites were cultured in erythrocytes using routine methods. Ring stage parasites were mixed at a 50/50 ratio between 2% to 4% haematocrit and 1-1.5% parasitaemia with growth (rings) counted 3 or 5 days post setup by microscopy of Giemsa stained thin smears. Variations on the parasite mix experiments are described in Supplementary Methods.

Electroporation of RBCs and plasmid addition

pHGBrHrBl-1/2 plasmid (400 μg) (encodes Bsd) (Wilson et al., 2010) was added or transfected into RBCs (Fidock and Wellems, 1997) and added to cultures of trophozoite stage CS2idhfr⁹²⁰. CS2eBsd^{GFP}+CS2idhfr⁹²⁰ parasites served as positive control. Drugs Bs+WR (2.5 μg/ml and 5 nM respectively) were added rings.

Live fluorescence and immunofluorescence

For immunofluorescence assays iRBCs were fixed by standard methods (Volz et al., 2012). Cells were imaged on a Zeiss Elyra PS.1 SR-SIM platform (Carl Zeiss GmbH) or a Line-scan confocal Zeiss LSM 5 Live fluorescent microscope. Structured Illumination Microscopy was performed on a Deltavision OMX V4 Blaze 3D Structured Illumination Microscopy (3D-SIM) system (Applied Precision).

Fluorescent *in situ* hybridization and polymerase chain reaction (PCR)

FISH was carried out as described previously (Volz et al., 2012). FISH experiments were visualized using a Zeiss LSM 5 Live fluorescent microscope.

Optiprep gradient purification of exosome-like vesicles

Media components were fractionated by centrifugation ($250,000 \times g$, 18 h, 4°C) through a continuous 10-30% Optiprep (Axis-Shield) gradient. Fractions (1 ml) were collected from the top of the gradient for further analysis.

Atomic Force Microscopy

Transwell supernatants were imaged *in situ* after deposition on mica surfaces. AFM images were then analysed for number, diameter and height (Supplementary methods).

Electron Microscopy

Magnet-purified infected RBCs were fixed in 2% paraformaldehyde/PBS, treated with Equinatoxin II (10 mg) (Anderluh et al., 1996) refixed in 2% paraformaldehyde/0.0075% Glutaraldehyde/PBS and blocked with 1% bovine serum albumin/PBS as described previously (Jackson et al., 2007). Cells were incubated with antibody (rabbit anti-PfPTP2) and 6 nm gold-conjugated protein A (Aurion) and observed on a Phillips CM120 at 120 kV. Negative staining and cryo-TEM of purified vesicles from Optiprep fractions was performed as described previously (Coleman et al., 2012). TEM was performed using a Tecnai G² F30 (FEI, Eindhoven, NL) transmission electron microscope, operating at 300 kV (Bio21 Molecular Science and Biotechnology Institute, Parkville, VIC, Australia), with defocus between 10-16 μm , across 15,000x-39,000x magnification.

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Figure 1. Communication between parasites results in DNA dependent transfer of drug resistance and fluorescence.

(A) Giemsa smears after 5 days for 3D7edhfr^{GFP} (episomal *hdhfr* gene, WR resistant) + CS2eBsd^{GFP} (episomal *blastocidin-S-deaminase* gene, Bs resistant) compared to each cultured alone with Bs+WR. (B) 3D7edhfr^{GFP}+CS2eBsd^{GFP} parasites co-cultured in Bs+WR and ring stage parasitaemia determined after 5 days compared to 3D7edhfr^{GFP} and CS2eBsd^{GFP} parental controls. Bars are mean and standard error of the mean (SEM) of 3 experiments. (C) CS2idhfr⁹²⁰+CS2eBsd^{GFP} co-culture compared to CS2idhfr⁹²⁰ in RBCs electroporated (el) with a Bsd resistance plasmid or plasmid added to media in Bs+WR. Bars are mean and SEM of 3 experiments. (D) Fluorescence microscopy of live 3D7idhfr^{mCh} (mCherry) and CS2eBsd^{GFP} (GFP) co-cultured. Top panels: 3D7idhfr^{mCh} expression of mCherry (red) in nucleus. Second panel: CS2eBsd^{GFP} expression of GFP in cytoplasm. Bottom panels: Examples of

CS2eBsd^{GFP}+3D7idhfr^{mCh} co-cultures in Bs+WR after 5 days. DAPI stained images (blue) show nucleus and final image merged. (E) FISH showing plasmids transferred between parasites. Top panel, CS2eBsd^{GFP} hybridised with *bsd* (red) and *hdhfr* (green). Middle panel, 3D7edhfr^{GFP} hybridised with *bsd* (red) and *hdhfr* (green). Bottom panel, CS2eBsd^{GFP}+3D7edhfr^{GFP} co-cultured with Bs+WR and hybridised with *bsd* (red) and *hdhfr* (green). Each panel shown with DAPI stained nuclei (blue) and merged. (E) PCR genotyping of drug resistance (*hdhfr* and *bsd*) and fluorescent protein genes (*mCherry* and *gfp*) from 3D7idhfr^{mCh} (P1), CS2eBsd^{GFP} (P2) and 3D7idhfr^{mCh}+CS2eBsd^{GFP} in (M+) or absence (M-) of Bs+WR after 12 days (first panel). Second panel: gDNA from parental 3D7idhfr^{mCh} (P1) or CS2eBsd^{GFP} (P2) parasites in Bs+WR after 8 or 12 days.

Figure 2. Parasite communication and DNA transfer is mediated by factor(s) released in culture supernatant.

(A) Transwells with 3D7edhfr^{GFP} (WR resistant) and CS2eBsd^{GFP} (Bs resistant) in the insert (I) and bottom chamber (B) containing Bs+WR. Ring stage parasitaemia was determined after 5 days compared to 3D7edhfr^{GFP}+CS2eBsd^{GFP}. (B) 3D7idhfr^{mCh} (WR resistant) and CS2eBsd^{GFP} (Bs resistant) separated in insert (I) and bottom chambers (B) with Bs+WR. Ring stage parasitaemia was determined at 5 days compared to 3D7idhfr^{mCh}+CS2eBsd^{GFP} co-cultured. Bars are mean and SEM of 3 experiments. (C) PCR amplification of *gfp* confirms transfer of DNA. CS2idhfr⁹²⁰ and CS2eBsd^{GFP} separated in transwells with Bs+WR compared to controls for *hDHFR* and *GFP*. (D) Rescue of *P. falciparum* growth by cell-cell communication and plasmid transfer. Culture supernatant from 3D7edhfr^{GFP}+CS2eBsd^{GFP} (50, 100, 150 and 200 μ l) in insert rescued growth of 3D7edhfr^{GFP} ring stage recipient (100 μ l,

1.5% parasitaemia). 3D7edhfr^{GFP} was added to bottom chamber and parasite survival determined at 3 days. Bars, mean and SEM of 3 experiments. (E) Parasite communication and DNA transfer occurs within 1 hr and continues 10 hr post drug treatment. CS2eBsd^{GFP} (insert) separated from 3D7edhfr^{GFP} (bottom well) containing Bs+WR. CS2eBsd^{GFP} removed at different time points (0-24 hr) and survival of 3D7edhfr^{GFP} determined at 3 days. Bars, mean and range of two experiments. For 2D & E bars represent mean and SEM of three experiments with differences assessed using a paired t-test (* P≤0.05, ** P≤0.01).

Figure 3. Actin and microtubule inhibitors block intercellular communication in *P. falciparum*.

(A) Parasite communication and plasmid transfer occurs mostly in ring stages. 3D7idhfr^{mCh}+CS2eBsd^{GFP} were co-cultured at ring or trophozoite stage and selected on Bs+WR. Ring stage parasitaemia was counted at 5 days. Bars: mean and SEM of 3 experiments. (B) Parasite communication and plasmid transfer increases under drug stress. 3D7edhfr^{GFP} and CS2Bsd^{GFP} co-cultured in transwell inserts with (+drug) or without Bs+WR (-drug). After 24 hr co-culture inserts were removed and naïve 3D7edhfr^{GFP} recipient added to bottom transwell with fresh Bs+WR. Growth of recipient 3D7edhfr^{GFP} was assayed after 3 days. Bars: mean and SEM of 4 experiments. (C) Treatment of ring stage 3D7edhfr^{GFP}+CS2eBsd^{GFP} parasites in Bs+WR co-cultured for 20 hr with actin/microtubule inhibitors. LATA; Latrunculin A; LATB, Latrunculin B; PH, Phalloidin; JAS, Jasplakinolide; COL, Colchicine; SWIN, Swinholide; NOC, Nocadazole; CytoD, Cytochalasin D; ORY, Oryzalin; Heparin; PBS, Phosphate buffered saline; R1, R1 peptide. Data represent ring stages expressed as percentage of a parallel non-Bs/WR treated control at 5 days. Sub-inhibitory concentrations determined from growth inhibition curves of 20 hr treated

3D7edhfr^{GFP}+CS2eBsd^{GFP} parasites. (B) Actin inhibitor cytochalasin D and (C) the microtubule inhibitor oryzalin show dose dependent inhibition at 5 days following 20 hr of Cyto D/oryzalin treatment of 3D7edhfr^{GFP}+CS2eBsd^{GFP} co-cultures in Bs+WR. Data represent ring stages as percentage of non-Bs/WR treated but cytochalasin D or oryzalin treated control. Bars: mean and SEM of 3 experiments.

Figure 4. Visualisation and size of vesicles from *P. falciparum*-infected RBCs.

(A) Factor(s) enabling communication between parasites are >100 kDa. Parasite communication was abolished when Bs+WR treated 3D7edhfr^{GFP} parasites (within dish) were separated from CS2eBsd^{GFP} (within dialysis tubing). Bars: mean and range of two experiments. (B) AFM image of CS2 RBCs showing vesicles surrounding the cell (black arrows) and budding from cell membrane (white arrows). Scale bar 2 μ m. (C) AFM imaging of supernatants from media, RBCs, CS2eBsd^{GFP}+3D7edhfr^{GFP} (Mix), CS2eBsd^{GFP}+3D7edhfr^{GFP} in Bs+WR (Mix++) and CS2eBsd^{GFP}+3D7edhfr^{GFP} in Bs+WR+CytoD (Mix++CytoD). Ring stage parasites were mixed (50/50 ratio) at same haematocrit and parasitemia. Histograms of vesicle diameter (top) and height (middle) distributions. Average number and SEM of vesicles in 100 μ m² displayed. Data from four independent experiments in triplicate. Scale bar 5 μ m. (C) AFM images of two larger vesicles (Mix ++, Section C). Scale bar is 600 nm. (D) Profile of three vesicles (red line in panel C, Mix ++) plotting height and diameter.

Figure 5. Purification and communication activity of exosome-like vesicles.

(A) Representative experiment (of nine independent gradients) showing purification of exosome-like vesicles with Optiprep velocity gradient centrifugation. Communication activity of gradient fractions screened by incubating ring stage 3D7edhfr^{GFP} with dilution series of each gradient compared to controls with media+Bs and culture supernatant+Bs. Parasite growth was assessed by comparing

the number of free merozoites, expressed as a percentage of control, of newly invaded cultures between treatments. (B) AFM visualisation of vesicles in fraction 3 (top panels) and fraction 4 (bottom panels). Size bars are shown on top panels and are same for corresponding panel below. (C) Negative-staining TEM of fraction 4 contents (top panels). In bottom panels are negative-staining TEM of fraction 3 (left), cryo-TEM of fraction 4 (middle and right). Inset scale bars = 50 nm.

Figure 6. Communication between parasites is dependent on the Maurer's cleft vesicle- located protein PfPTP2.

(A) Cell-cell communication screening of *P. falciparum* knockout strains. CS2idhfr^{ΔPTP1}, CS2idhfr^{ΔPTP2}, and CS2idhfr^{ΔPTP3} have gene disruptions blocking PfEMP1 trafficking to parasite-infected RBCs. Knockout lines co-cultured with donor CS2eBsd^{GFP} for 5 days in Bs+WR. Final parasitaemia expressed as percentage of CS2idhfr⁹²⁰. Bars: mean and SEM of three experiments. (B) Localisation of PfPTP2 in CS2idhfr^{PTP2/HA}-infected RBCs. PfPTP2, green, first panel; SBP1 (Maurer's cleft marker), red, second panel; DAPI, blue, third panel; all panels merged, fourth panel. (C) Immuno-EM of CS2idhfr^{PTP2/HA}-infected RBCs after treatment with equinotoxin II. MC, Maurer's cleft; RBCM, red blood cell plasma membrane; PVM, parasitophorous vacuole membrane. Panel a. Side panel shows higher magnification of Maurer's cleft. Arrow point to budding vesicle where PfPTP2 localised. Panel b. Arrows point to PfPTP2 on budding vesicle and membrane material. Panel c. Example of budding vesicle with PTP2 localisation. (D) Comparison of vesicle release from CS2idhfr^{ΔPTP1} and CS2idhfr^{ΔPTP2} by AFM. Quantity of vesicles released into transwell supernatants shown in corresponding histogram. Scale bar 2 μm.

Figure 7. Communication between parasites results in differentiation to sexual stages and transfer of DNA.

(A) Fluorescence microscopy of gametocytes produced by 3D7idhfr^{mCh}+3D7iBsd^{CK1-GFP} in Bs+WR. (B) Gametocytaemias from 3D7edhfr^{GFP} or CS2eBsd^{GFP} alone and 3D7edhfr^{GFP}+CS2eBsd^{GFP} co-cultured. (C) Gametocytaemias from 3D7iBsd^{CK1-GFP} or 3D7idhfr^{mCh} alone and 3D7iBsd^{CK1-GFP}+3D7idhfr^{mCh} co-cultured with Bs+WR. Microscopy counts expressed as percent gametocytes of uninfected RBCs (D) Flow cytometry of gametocytaemia for Bs+WR treated 3D7edhfr^{GFP} and CS2eBsd^{GFP} parental controls, 3D7edhfr^{GFP}+CS2eBsd^{GFP} co-cultured and 3D7idhfrBsd^{175/181} double drug resistant control. Gametocytes measured are from 3D7edhfr^{GFP} (data not shown). Gametocytaemia for the 50/50 3D7edhfr^{GFP}+CS2eBsd^{GFP} co-culture has been calculated to reflect the contribution of the 3D7edhfr^{GFP} line. Gametocytaemia expressed as percentage of 3D7iWRBsd^{175/181} control for 200,000 RBCs. Bars: mean and SEM of 3 or more experiments. (E) Giemsa smears of sorted NF54 gametocytes confirmed gating of gametocytes by flow cytometry. (F) Representative flow cytometry plots with gated gametocyte population (square gate) for 3D7edhfr^{GFP} and CS2eBsd^{GFP} controls, 3D7edhfr^{GFP}+CS2eBsd^{GFP} co-culture compared to 3D7idhfrBsd^{175/181} on Bs+WR.

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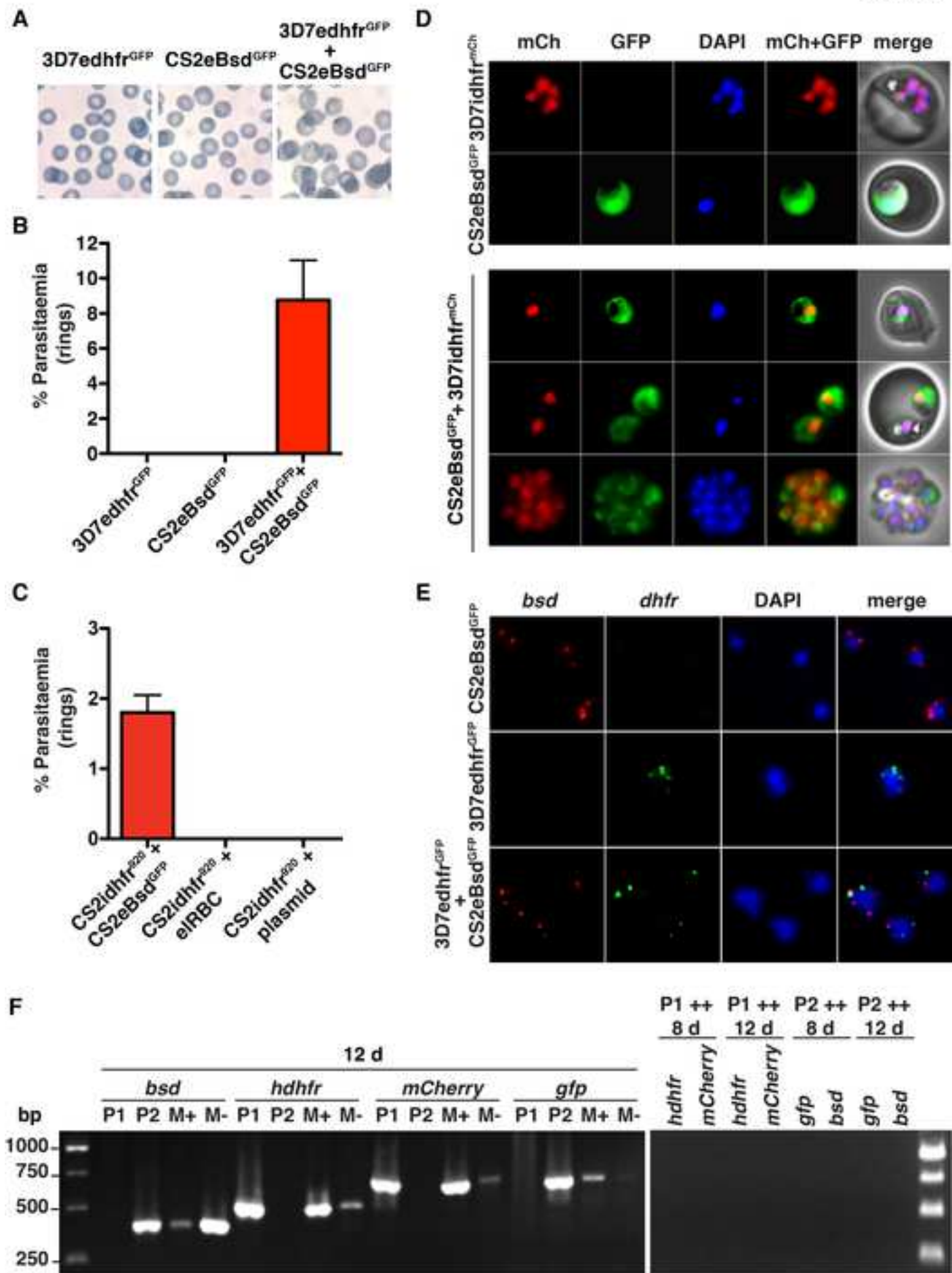


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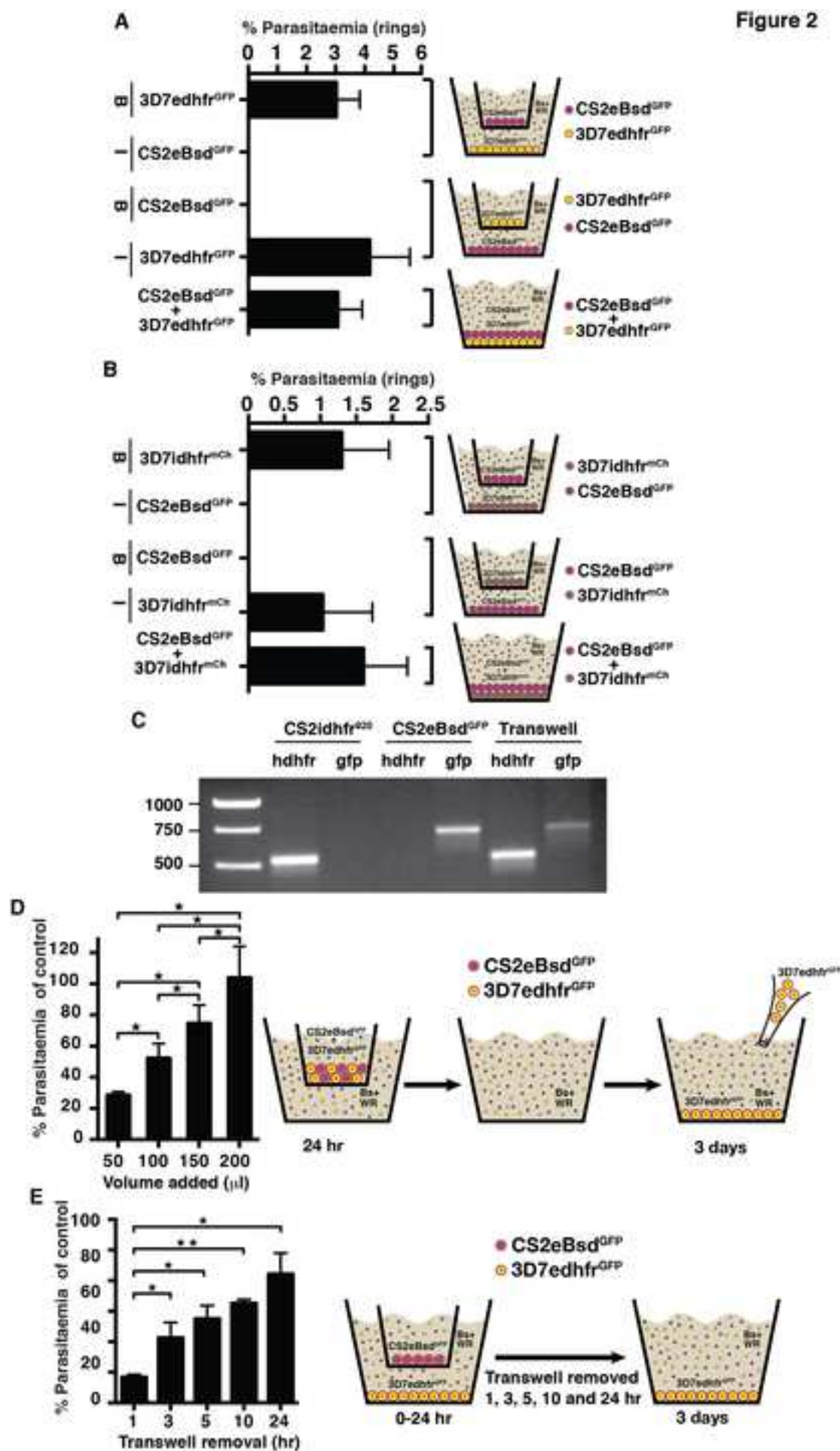


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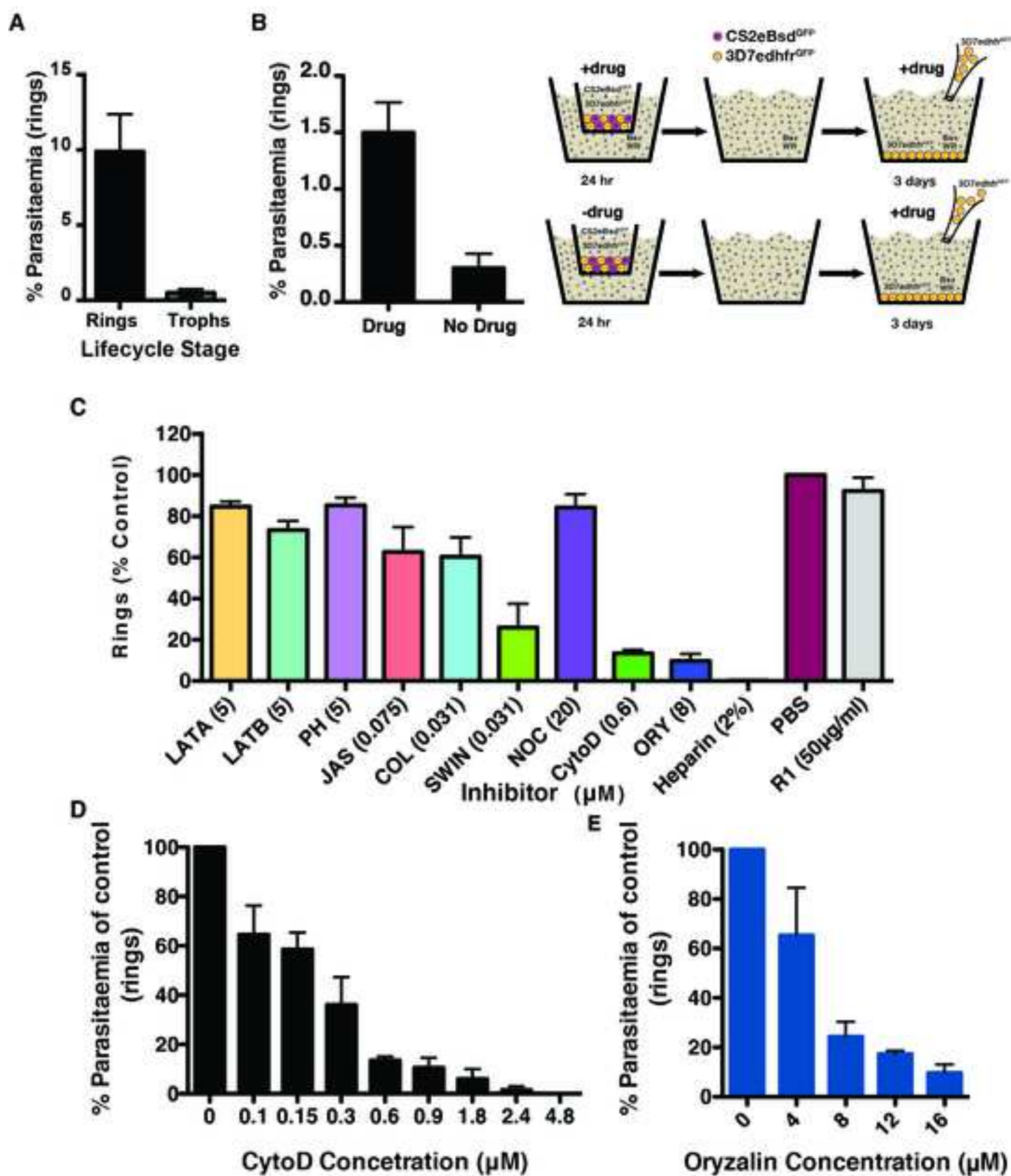


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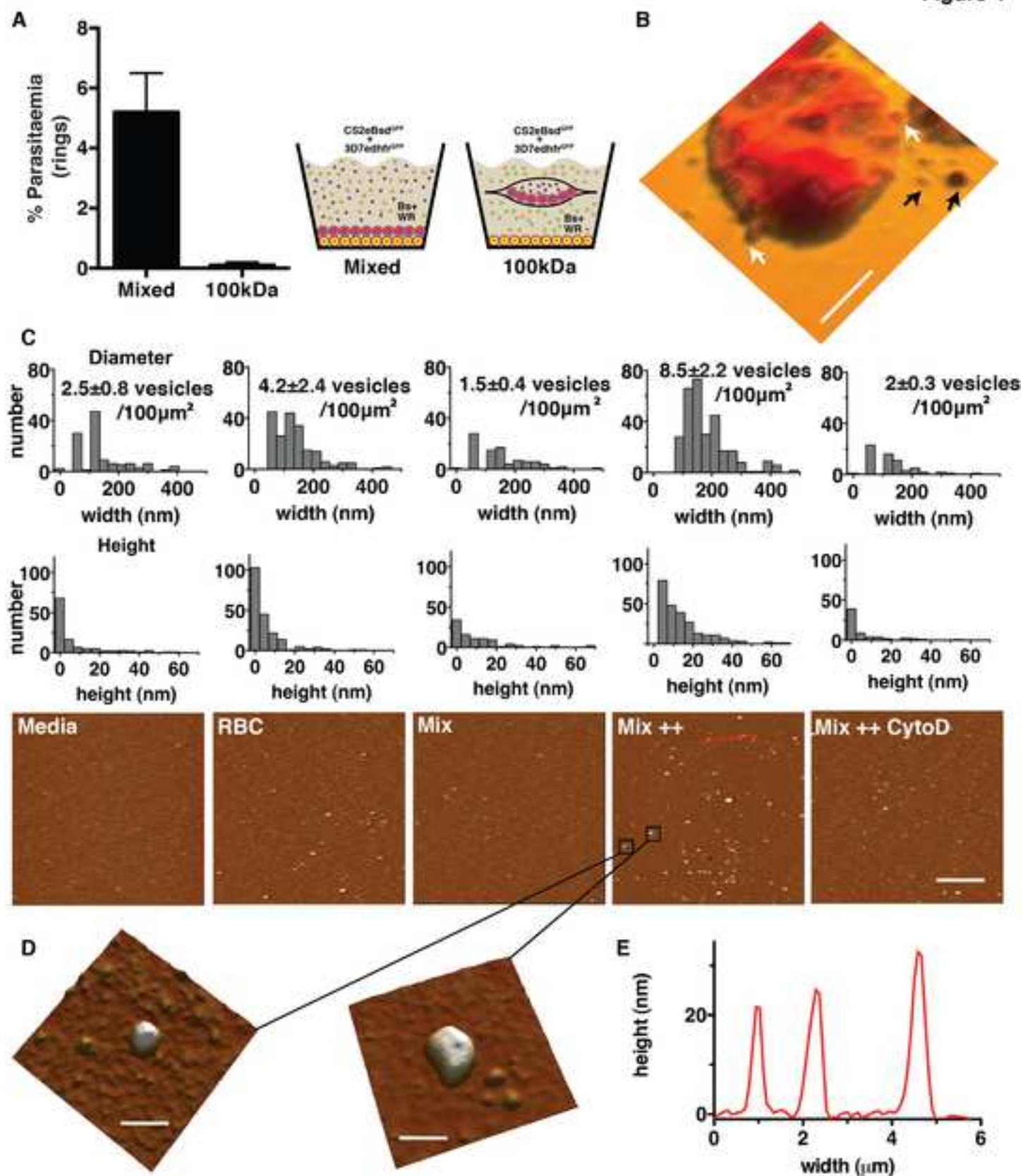


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Fig. 5

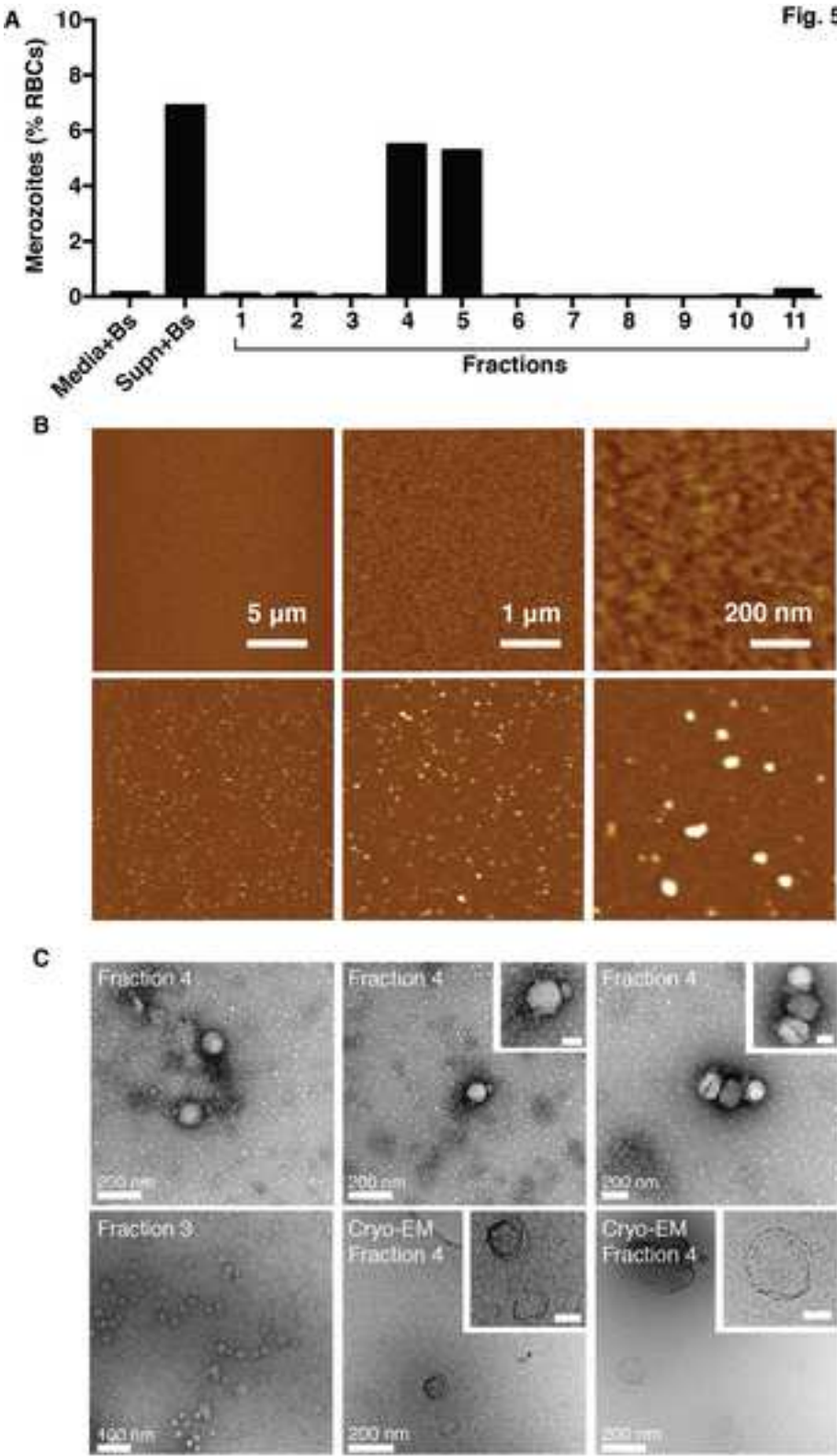


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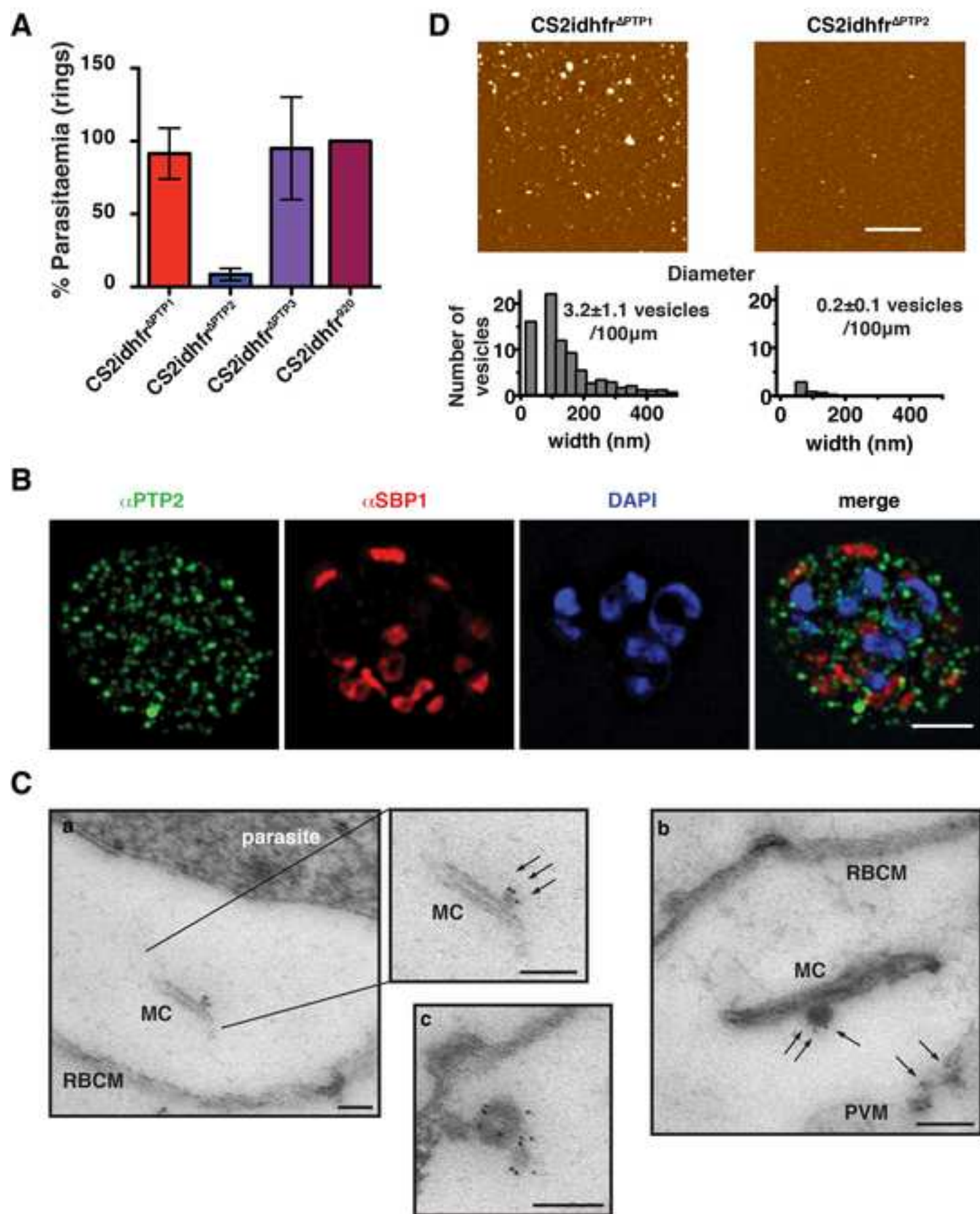
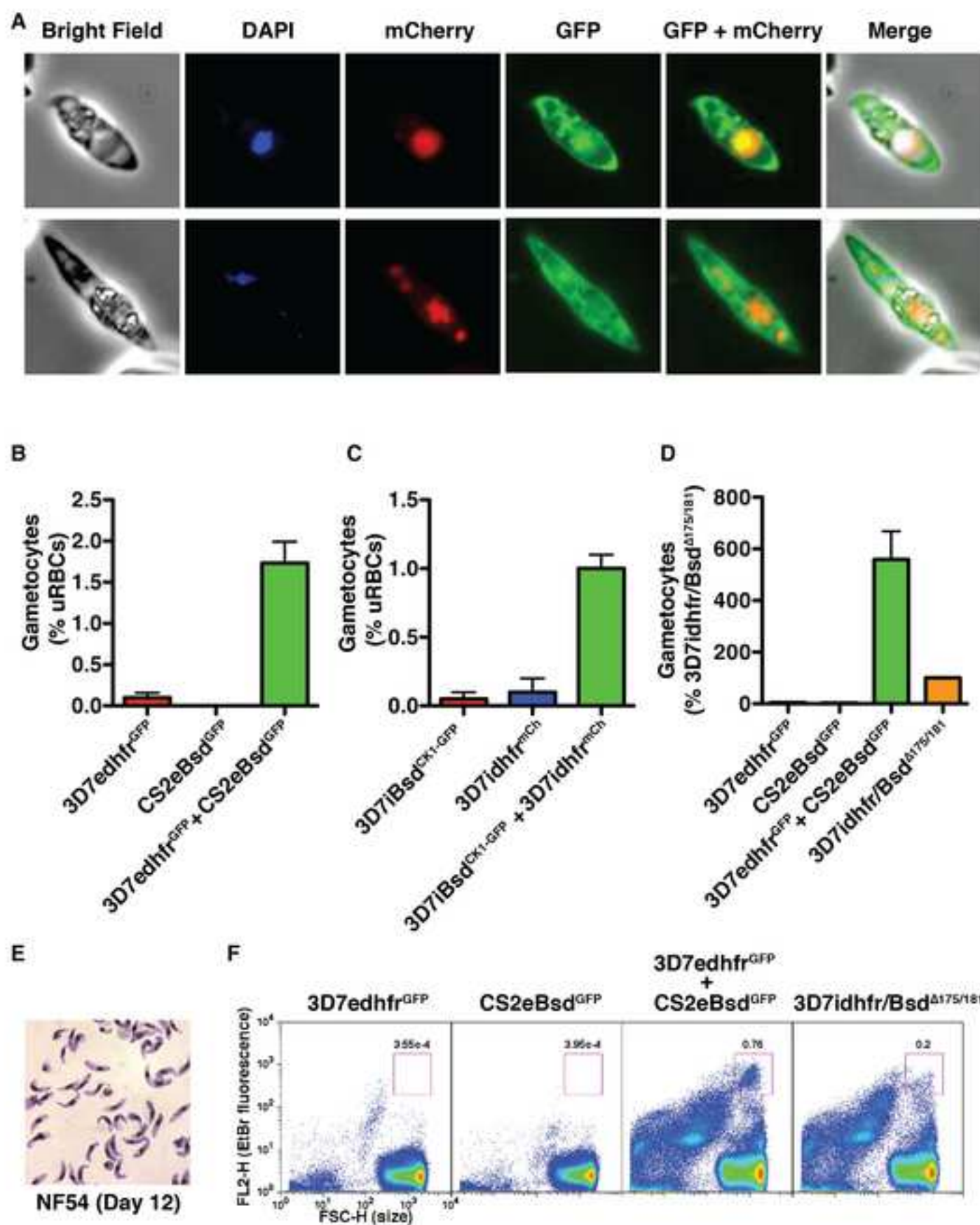


Figure 7
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Figure 7



Supplementary Inventory.

Supplementary Figures.

Figure S1. Bs acts predominantly against rings and early trophozoites.

Figure S2. Transfer of drug resistance markers occurs equally as well using mixed or single line supernatants.

Figure S3. Characterisation of *ptp2* gene disruption and HA-tagged CS2 *P. falciparum* lines.

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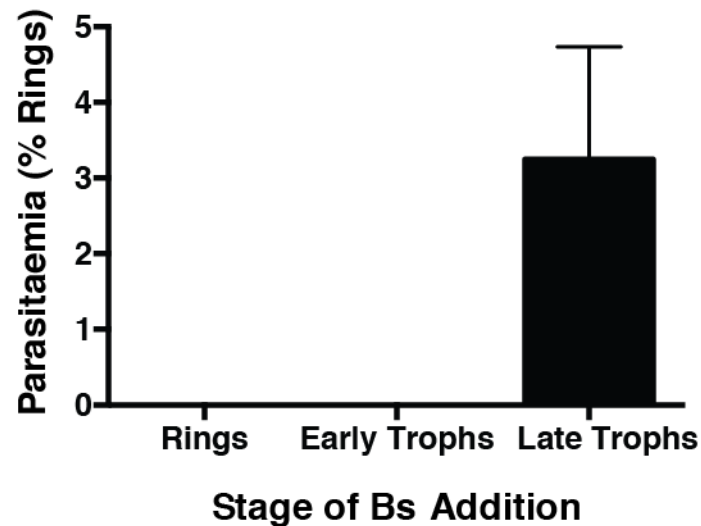
Supplementary Material.**Supplementary Figures.****Fig. S1**

Fig. S1. Bs acts predominantly against rings and early trophozoites. 3D7edhfr^{GFP} parasites were treated with Bs at early ring stage (approximately 0-8 hour post invasion), early trophozoite stage (20-28 hour post invasion) and late trophozoite stage (30-38 hour post invasion). Parasitaemia was assessed by microscopy counts of ring stage parasites 48 hours post assay setup. Treatment at either early ring or early trophozoite stages abolished parasite growth, whereas treatment at late trophozoite stages had greatly reduced inhibition of ring stages. Data represent the mean and range of two independent experiments.

Fig. S2

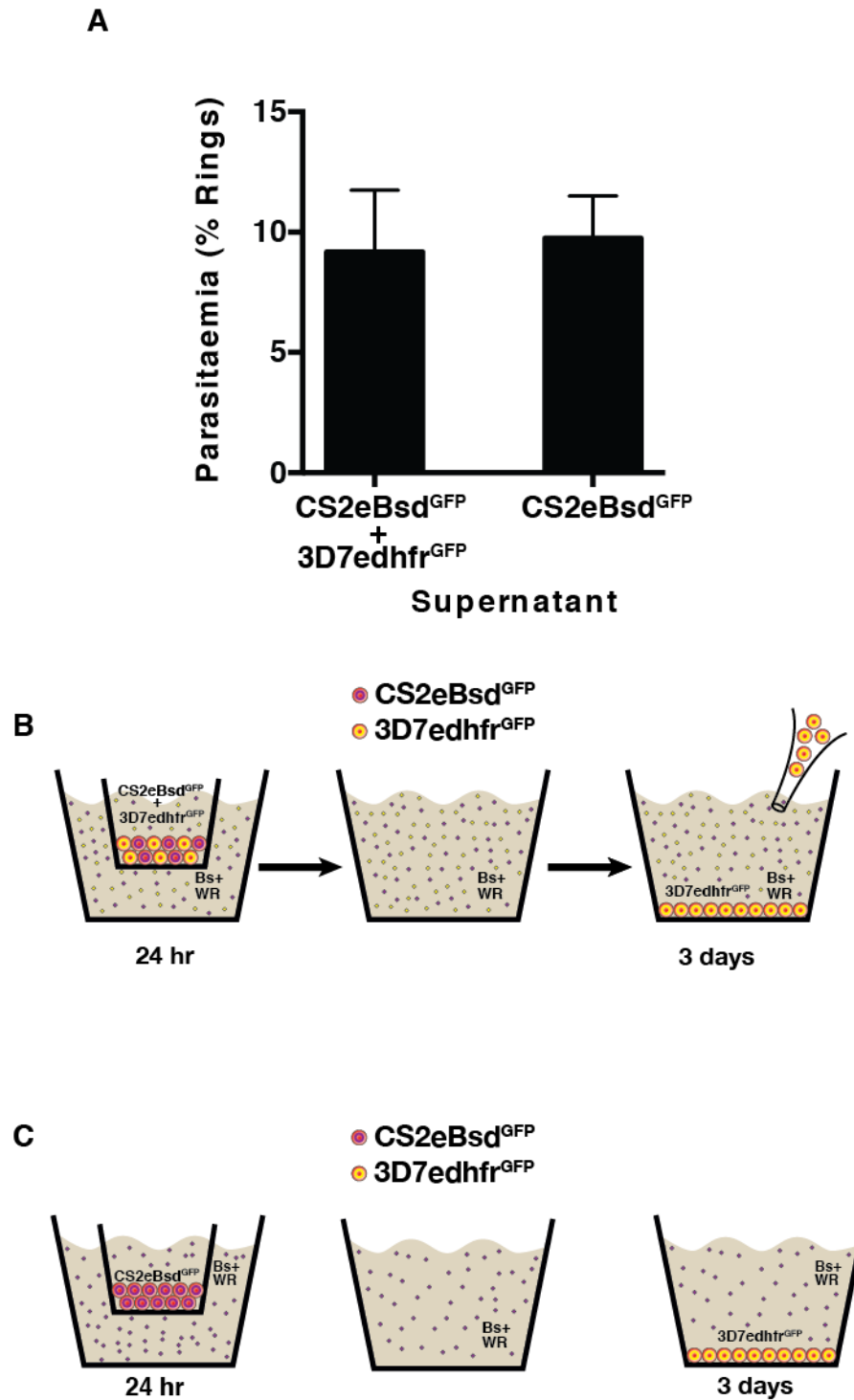


Fig. S2. Transfer of drug resistance markers occurs equally as well using mixed or single line supernatants. (A) The ability of supernatants collected from co-cultures of CS2eBsd^{GFP} and 3D7edhfr^{GFP} (with Bs+WR) or collected from single cultures of the CS2eBsd^{GFP} line (with Bs+WR) to rescue the Bs sensitive 3D7edhfr^{GFP} line was tested in parallel. Supernatant from the co-culture or single culture (Bs donor line only) experiment both showed equally high rates of rescue of the Bs sensitive

3D7edhfr^{GFP} line. Data represents the mean and SEM of 3 experiments. Schematics show how the co-culture (B) and single culture (C) experiments were undertaken. The insert containing ring stage co-culture or single cultures was removed after 24 hr and the Bs sensitive 3D7edhfr^{GFP} line was added. Parasitaemia of the 3D7edhfr^{GFP} line was assessed by microscopy counts of ring stage parasites 48 hr after addition to the supernatant.

Fig. S3

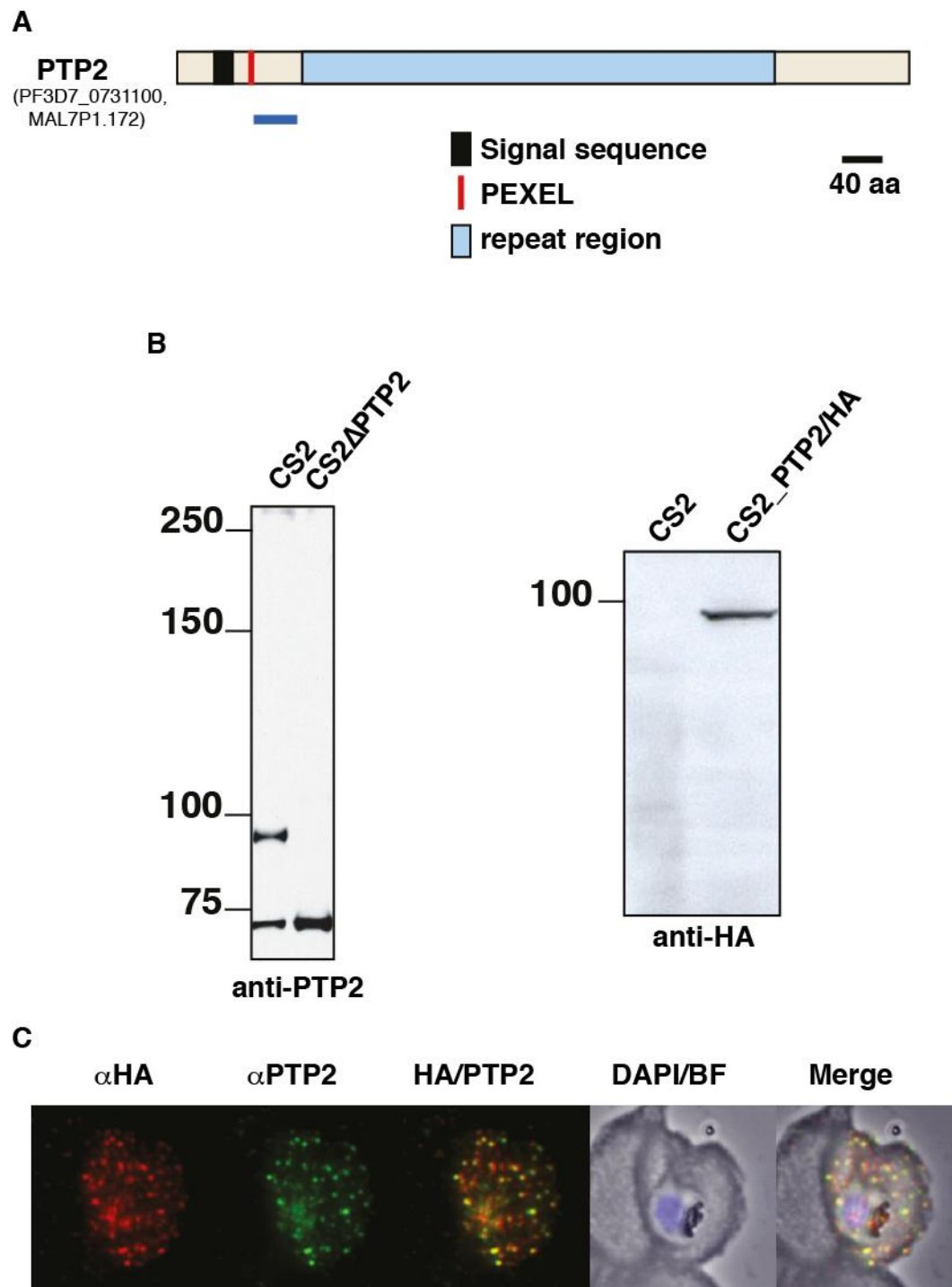


Fig. S3. Characterisation of *ptp2* gene disruption and HA-tagged CS2 *P. falciparum* lines. (A) Structure of the PTP2 protein. Shown is the signal sequence (black), PEXEL (Plasmodium export element, red) (Hiller et al., 2004; Marti et al., 2004), repeat region (blue) with size bar in amino acids. The antibodies used were made to amino acids 117-130 as shown by a blue bar. (B) Left panel is a western blot of the CS2 Δ idhfr^{PTP2} *P. falciparum* line in which the *ptp2* (MAL7P1.172) gene has been

disrupted (Maier *et al.*, 2008). The band at approximately 70 kDa is a cross-reactive protein to the anti-PTP2 antibodies. The right panel is a western blot of CS2idhfr^{PTP2/HA} in which the *ptp2* gene has been tagged at the 3' end by a sequence encoding haemagglutinin epitopes (HA) compared to the parental CS2 parasite line. (C) Immuno-fluorescence of CS2idhfr^{PTP2/HA} parasites using anti-PTP2 and anti-HA antibodies to show that both co-localise.

Fig. S4

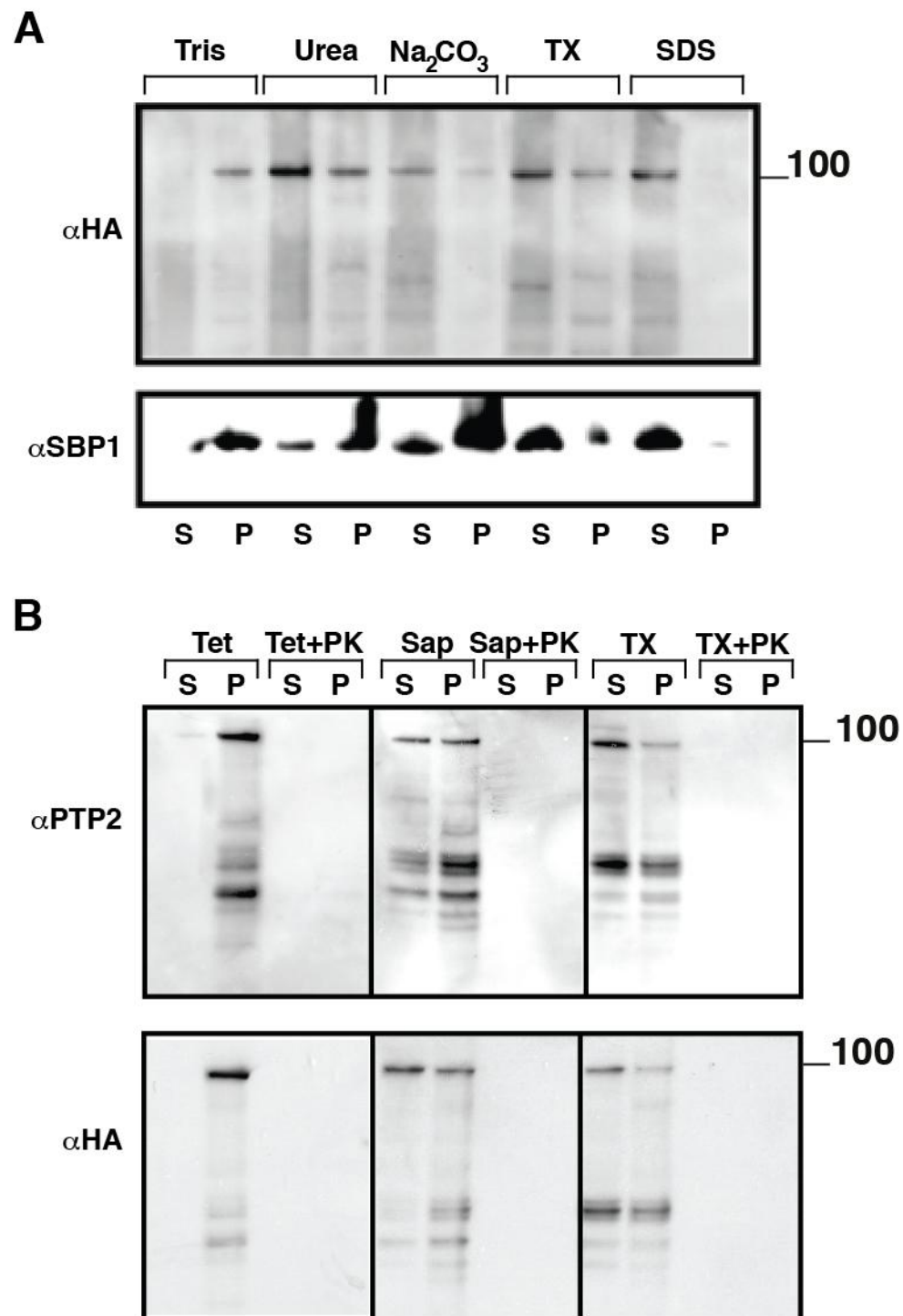


Fig. S4. PfPTP2 is present on the outside of Maurer's clefts and small vesicles bound to lipid or a protein(s). (A) Solubilisation of PfPTP2 in Tris.HCL, Urea, Na₂CO₃, Triton X100 and SDS. The upper panel was probed with anti-HA to detect the HA-tagged PfPTP2 from CS2idhfr^{PTP2/HA} parasites. Lower panel is probed with anti-SBP1 antibodies. SBP1 is a type 1 transmembrane protein that is inserted into the

membrane of Maurer's clefts (Cooke et al., 2006; Maier et al., 2007). (B) PTP2 is sensitive to proteinase K after tetanolysin-treatment of CS2idhfr^{PTP2/HA}-infected RBCs. Shown are parasites treated with tetanolysin (Tet), saponin (Sap) or Triton X100 (TX) followed by proteinase K (PK). The top panels are probed with anti-PTP2 antibodies and the bottom panel with anti-HA antibodies to detect PTP-HA.

Supplementary Materials and Methods.

Parasite lines

Genetically modified *P. falciparum* lines included: CS2eBsd^{GFP}. This parasite line has an episomal plasmid encoding cytosolic GFP and Bsd (Ataide et al., 2010). 3D7edhfr^{GFP}: This parasite line has an episomal plasmid encoding hDHFR and GFP. The GFP protein is expressed as a fusion protein with PfEMP3, PF3D7_0201900 (Boddey et al., 2010). 3D7idhfr^{mCh}: This parasite line has an integrated plasmid that fuses mCherry with the 3' end of PF3D7_0919000 (Volz et al., 2010). This protein is localised to the nucleus (Volz et al., 2010). 3D7iBsd^{CK1-GFP}: This parasite line has a plasmid that has integrated into the gene encoding CK1 (GFP fused to CK1, PF3D7_1136500) and was a kind gift from Dominique Dorin-Semblat and Christian Doerig, unpublished. CS2idhfr⁹²⁰: This parasite line has *hdhfr* inserted so that it deletes and disrupts the gene PFB0920w by double crossover recombination (Maier et al., 2008). CS2idhfr^{ΔPTP1}: This parasite line has *hdhfr* inserted so that it deletes and disrupts the gene PFB0106c (PfPTP1) by double crossover recombination (Maier et al., 2008). CS2idhfr^{ΔPTP2}: This parasite line has *hdhfr* inserted so that it deletes and disrupts the gene MAL7P1.172 (PfPTP2) by double crossover recombination (Maier et al., 2008). CS2idhfr^{ΔPTP3}: This parasite line has *hdhfr* inserted so that it deletes and disrupts the gene PF14_0758 (PfPTP3) by double crossover recombination (Maier et al., 2008). 3D7idhfr/Bsd^{175/181}: This parasite line has *hdhfr* inserted into the *eba175* gene by double crossover recombination so that it deletes and disrupts the gene. Additionally, it has *bsd* inserted into the *eba181* gene by double crossover recombination so that it deletes and disrupts the gene (Lopaticki et al., 2011). CS2idhfr^{PTP2/HA}: This parasite line has a plasmid inserted into the 3' end of the *ptp2* gene (MAL7P1.172) so that the PTP2 protein was tagged with HA epitopes (Fig. S3).

P. falciparum asexual stage parasites were maintained in culture in human O⁺ erythrocytes at 4% haematocrit in RPMI-HEPES supplemented with 0.5% (w/v) AlbumaxTM (Invitrogen) as previously described (Trager and Jensen, 1976). Parasites were synchronised by sorbitol lysis selection of ring stage parasites. The basic mixing experiments involved co-culture of ring stage parasites at a 50/50 ratio between donor and recipient lines between 2-4% haematocrit and 1-1.5% parasitaemia (Fig. 1 A-F, Fig. 6 A). Parasite knockout lines were co-cultured with donor CS2eBsd^{GFP}, for 5 days in Bs+WR. Final parasitaemia (ring stage) expressed as percentage of CS2idhfr⁹²⁰ control (Fig 6 A)

Mixing experiments

Transwell experiments: Transfer of drug resistance across a 0.4 µM membrane was assessed using 12 mm diameter (800 µl volume) and 75 mm diameter (15 ml) transwells (Corning) with ring stage parasitaemia counted 3-5 days post mixing by microscopy (Fig. 2 A & B). A number of variations on the standard transwell experiments are described in the results including removal or addition of single lines in a well within 24 hours of mixing, addition of drug after removal of donor line, collection of supernatant and subsequent transfer of drug resistance in supernatants (Fig. 2 D&E, Fig. 3B, Fig. S2). Supernatant collected from transwells were also used for AFM assessment of vesicles (Fig. 4B-E, Fig. 6 D) and purification using density gradients (Fig. 5 A-C).

100 kDa Dialysis Tubing experiments: Transfer of drug resistance across a 100 kDa cut-off cellulose acetate dialysis membrane (Spectrum) was assessed for the CS2eBsd^{GFP} (inside dialysis tubing) and 3D7edhfr^{GFP} (outside dialysis tubing) lines under selection of both drugs (Fig. 4 A). Dialysis tubing was washed in water overnight, sterilised with 50% Ethanol for 20 minutes and then washed in growth medium.

Actin and microtubule inhibitor experiments: Sub-lethal concentrations of microtubule and actin inhibitors on parasite growth were determined by treatment of a mixed culture of ring stage parasites with a dilution series of the inhibitors, washing of the culture 20 hours post setup and then assessment of late stage parasitaemia by flow cytometry 48 hours later. Inhibition of communication with microtubule and actin inhibitors was examined in more detail through treatment of co-cultured CS2eBsd^{GFP} and 3D7edhfr^{GFP} parasites with and without Bs+WR with sub-lethal concentrations of

the individual inhibitors for 20 hours starting at early ring stage (Fig. 3 C-E). The inhibitors were washed out and the antimalarials added back into the culture where appropriate. Ring stage parasitaemia was counted 5 days post mixing and the effect of actin and microtubule inhibitors on communication between drug treated co-cultures was expressed as a percentage of the non-drug treated control.

Electroporation of RBCs and free plasmid addition: To test non-specific transfer of plasmids in mixing experiments, 400 µg of the Bs selectable cytosolic GFP expressing pHGBrHrBI-1/2 plasmid (Wilson et al., 2010) was transfected (Crabb and Cowman, 1996) into uninfected RBCs or added to culture media (10 ml) prior to addition of CS2idhfr⁹²⁰ parasites (Fig. 1 C). A mix of the CS2eBsd^{GFP} and CS2idhfr⁹²⁰ parasite lines served as the positive survival control after addition of Bs+WR to all treatments. The CS2eBsd^{GFP} and CS2idhfr⁹²⁰ lines were added at 1% late trophozoite stage to allow invasion into electroporated RBCs within 24 hours. Electroporated RBCs were left to rest under standard culture conditions for 4 hours prior to addition of parasites and then added to cultures such that 40% of the total RBCs had been electroporated.

Assessment of gametocytogenesis: Asexual stage parasites were depleted in drug treated cultures from day 6 post mixing for the assessment of gametocytes using N-acetyl glucosamine (NAG) (Ponnudurai et al., 1986). Gametocytaemia was assessed 10-12 days post mixing by microscopy of Giemsa stained thin smears, fluorescence microscopy or flow cytometry (stained with 100 µg/ml EtBr) (Fig. 7 A-F). Flow cytometry data was analysed using FlowJo software (Treestar Inc.). Cell sorting, using a (FACSAria, Becton Dickinson), of a FL2 high (EtBr fluorescent), FSC high (size) population confirmed that the gated population measured by flow cytometry was predominantly mature gametocytes.

Live fluorescence and immunofluorescence assays

Fluorescence microscopy of cell sorted drug treated asexual stage (6 days post mixing, Fig. 1 D) and gametocyte stage (10-12 days post mixing, Fig. 7 A) parasites to identify transfer of fluorescence marker was completed on a Zeiss LSM 5 Live fluorescent microscope. For immunofluorescence assays, iRBCs were fixed according to standard methods (Tonkin et al., 2004). Cells were imaged on a Zeiss Elyra PS.1 SR-SIM platform (Carl Zeiss GmbH). Alternatively, Structured Illumination

Microscopy was performed on a Deltavision OMX V4 Blaze 3D Structured Illumination Microscopy (3D-SIM) system (Applied Precision) (Fig. 6 B).

Immunofluorescence assays for Fig. S3 were performed as described (Rug et al., 2006) Briefly, acetone/methanol (90%/10%) fixed smears of CS2 and CS2idhfr^{PTP2/HA} infected erythrocytes were probed with either mouse (Mab) anti-HA (1:100) or rabbit anti-PTP2 (1:200) antiserum. Secondary antibodies were Alexa-Fluor 488-conjugated anti-rabbit IgG (Molecular Probes) and Alexa-Fluor 594-conjugated anti-mouse IgG (Molecular Probes). Cells were viewed with a Zeiss Plan-Apochromat 100x/1.4 numeric aperture oil-immersion lens on a Zeiss Axioskop 2 microscope equipped with a PCO SensiCam (12 bit) camera and Axiovision 4 software (Zeiss). Captured images were processed using Photoshop and ImageJ software (<http://rsb.info.nih.gov/ij>).

Fluorescent *in situ* hybridization and polymerase chain reaction (PCR)

DNA-FISH was carried out on mix-stage parasites as described previously (Dzikowski et al., 2007) for labelling of *bsd* (biotin High-Prime Kit, Roche; streptavidin AlexaFluor 594 secondary, Invitrogen) and *hdhfr* (Dig High-Prime Kit, Roche; mouse anti DIG primary, Sigma; rabbit anti mouse 488 secondary, Invitrogen) plasmid DNA (Volz et al., 2012) (Fig. 1 E). FISH experiments were visualized using a Line-scan confocal Zeiss LSM 5 Live fluorescent microscope. Genomic DNA for PCR was extracted as previously described (Triglia and Cowman, 1994).

Atomic Force Microscopy

We cultured different parasite lines and controls in the insert of transwells (400 μ M filter, 800 μ l volume) (Corning) and the vesicles released into the bottom compartment were analysed by AFM. For Fig. 4 C each experiment was set up as outlined in the following table.

	Media	RBC	Mix	Mix ++	Mix ++ CD
Insert	Media (1)	100 μ l washed packed RBCs in Media (1)	100 μ l washed packed RBCs+ CS2eBsd ^{GFP} (2) at 0.75% parasitaemia	100 μ l washed packed RBCs+ CS2eBsd ^{GFP} (2) at 0.75% parasitaemia	100 μ l washed packed RBCs+ CS2eBsd ^{GFP} (2) at 0.75%

			+ 3D7edhfr ^{GFP} (2) at 0.75% parasitaemia in Media (1)	+ 3D7edhfr ^{GFP} (2) at 0.75% parasitaemia in Media (1) + WR (3) + Bs (4)	parasitaemia + 3D7edhfr ^{GFP} (2) at 0.75% parasitaemia in Media (1) + WR (3) + Bs (4) + CytoD (5)
Bottom	Media (1)	Media (1)	Media (1)	Media (1) + WR (3) + Bs (4)	Media (1) + WR (3) + Bs (4) + CytoD (5)

Each number in the table refers to the following additions to the relevant culture well.

- (1) RPMI-HEPES supplemented with 0.5% (w/v) AlbumaxTM (Invitrogen) as previously described (Trager and Jensen, 1976)
- (2) RBCs infected with either CS2eBsd^{GFP} or 3D7edhfr^{GFP} lines cultured at 4% haematocrit were collected at 1.5% ring stage parasitaemia and counted 3-5 days post mixing by microscopy. All independent experiments were normalized so that CS2eBsd^{GFP} and 3D7edhfr^{GFP} parasite lines, co-cultured for Mix, Mix ++ and Mix ++ CD conditions, were at the same haematocrit and parasitemia and taken from the same culture dish.
- (3) 0.2 µl of WR (5 nM)
- (4) 0.5 µl of Bs (2.5 µg/ml)
- (5) 0.7 µl Cytochalasin D (0.6 µM)

Transwell supernatants from the bottom of the five conditions detailed above were collected after 10 hours and a sample (100 µl) deposited on 0.1% polyethyleneimine (PEI) coated mica surfaces (Muscovite mica, ProSciTech) and imaged without washing or drying with an MFP3D-BIO atomic force microscope (Asylum Research) (Fig. 4 B-E, Fig. 5 D, Fig. 6 B). Samples were imaged in acoustic mode using silicon nitride probes (nominal spring constant 0.1 N/m, resonant frequency 38 kHz, Bruker AFM Probes).

Four independent experiments were set up as described above for each of the five conditions. In each independent experiment 3 scans ($20 \times 20 \mu\text{m}^2$, 1 Hz scan rate, 256 lines $\times$ 256 points) taken at 3 different random points of the surface were analysed for number, width and height using the “flooding” function of the WSxM software (Nanotec) (Horcas et al., 2007). This function allowed determination of vesicle width and height histograms, shown in Figure 4 C, corresponding to 12 different scans. From histograms the number of vesicles could be obtained which was related to the surface area (12 times $20 \times 20 \mu\text{m}^2$) as a measure of the vesicle surface distribution.

For Fig. 6 D each experiment was set up as outlined in the following table to quantitate the number of vesicles released from CS2idhfr^{ΔPTP1} and CS2idhfr^{ΔPTP2}.

	CS2idhfr ^{ΔPTP1}	CS2idhfr ^{ΔPTP2}
Insert	100 μl of packed RBCs + CS2idhfr ^{ΔPTP1} (2) at 1.5 % parasitemia in Media (1)	100 μl of RBCs + CS2idhfr ^{ΔPTP2} (2) at 1.5 % parasitemia in Media (1)
Bottom	Media (1)	Media (1)

Each number in the table refers to the following additions to the relevant culture well.

- (1) RPMI-HEPES supplemented with 0.5% (w/v) AlbumaxTM (Invitrogen) as previously described (Trager and Jensen, 1976)
- (2) RBCs infected with either CS2idhfr^{ΔPTP1} or CS2idhfr^{ΔPTP2} lines cultured at 20% haematocrit were collected at 1.5% ring stage parasitaemia counted 3-5 days post mixing by microscopy.

Supernatants for quantifying vesicle release were taken from transwell cultures after 10 hours and deposited on mica surface for AFM characterization as for Figure 4 C. In the same way, 4 independent experiments were set up and 3 random scans taken and analysed for number, as well as width and height frequencies for each independent experiment. In this case, we set up the experiment with the two single lines at 10 times higher haematocrit in order to quantify the very low number of vesicles released by CS2idhfr^{ΔPTP2}. Hence, number, width and height quantification for both parasite lines was normalized for Figure 4 C.

Optiprep gradient purification

Media (2 ml) was overlaid on a 10-ml continuous 10-30% OptiprepTM (Axis-Shield) gradient made up in TBS (140 mM NaCl, 10 mM Tris, pH 7.4) and centrifuged ($250,000 \times g$, 18 hours, 4°C , in a SW41 rotor; Beckman Coulter). Fractions (1 ml) were collected from the top for further analysis (Fig. 5 A-C). Functional activity of gradient fractions was assessed by incubating ring stage 3D7edhfr^{GFP} parasites with a dilution series of each gradient (25-6.25% final concentration) and growing the parasites for a further 48 hours. Transfer of Bs resistance plasmid was assessed by screening the cultures for the number of free merozoites according to the method of (Wilson *et al.*, 2013) and confirming the presence of rings by microscopy. Briefly, exosome-vesicle like treated cultures were stained with EtBr at ring stage and screened by flow cytometry. The low FSC (size) high FL2 (EtBr fluorescent) cells corresponding to free merozoites were gated along with the RBC population. The number of free merozoites was expressed as a percentage of the number of RBCs counted. Using this method it was possible to rapidly screen for successful development and rupture of schizonts of Bs treated cultures with subsequent invasion confirmed by microscopy.

Electron microscopy

A glow-discharged 300 mesh Cu grid coated with Formvar film (ProSciTech) was floated on a 20 μl aliquot of the particular OptiprepTM fraction for 5 min, followed by washing 2 x TBS and stained with 1.5% uranyl acetate. Transmission electron microscopy was performed using a Tecnai G² F30 (FEI) transmission electron microscope operating at 300 kV (Bio21 Molecular Science and Biotechnology Institute, Parkville, VIC, Australia), with defocus between 10-16 μm , across $15,000\times - 39,000\times$ magnification. Electron micrographs were captured with a Gatan UltraScan[®] 1000 2k $\times$ 2k CCD camera (Gatan, Inc.) (Fig. 6 C).

Cryo-Electron microscopy

A 3 μl aliquot of the suspension was transferred onto glow discharged lacey-hole carbon grids (ProSciTech). After blotting of excess liquid, grids were plunge frozen in liquid ethane cooled by liquid nitrogen, using a manual plunger. Grids were mounted in a Gatan cryoholder (Gatan, Inc.) in liquid nitrogen. Images were acquired at 300 kV using a Tecnai G² F30, in low dose mode and $20,000 \times$ magnification (Fig. 5 C).

Solubility Assay

In solubility assays, CS2idhfr^{PTP2/HA} infected RBCs were subjected to haemolysis in H₂O with protease inhibitors (Complete Mini; Roche) (Fig. S4). Pelleted cell lysates were subjected to treatment with the following reagents (including complete protease inhibitors: 1) 100 mM Na₂CO₃ pH 11.5 for 1 hr on ice; 2) 6 M urea in 10 mM Tris-HCl pH 8 for 1 hour at room temperature; 3) 2% Triton X-100, 0.2M SB201 (Sigma) in 10 mM Tris-HCl pH 8 for 1 hour at room temperature; 4) 2% SDS in 10 mM Tris-HCl pH 8 for 1 hour at room temperature and 5) 10 mM Tris-HCl pH 8 for 1 hour at room temperature. After washing, supernatants and pellets were taken up in SDS-PAGE loading buffer (Invitrogen) and subjected to SDS-PAGE/Western Blot analysis. Blots were probed with mouse anti-HA antibodies (Roche; 1:1000), stripped (BioRad) and probed with anti-SBP1 (rabbit; 1:500).

Membrane Orientation Assay

CS2idhfr^{PTP2/HA} infected RBCs (10⁸) were treated with 100 U/mL tetanolysin (Sigma) in 0.2% Bovine Serum Albumin (BSA; Sigma) containing protease inhibitors (complete Mini, Roche) for 20 min at 37°C. After washing, 6 aliquots of the samples were treated with the following reagents: 1) 50 µl PBS; 2) 50 µl 100 µg/ml Proteinase K (Sigma) in PBS; 3) 50 µl 0.09% saponin (Kodak); 4) 50 µl 0.09% saponin containing 100 µg/ml Proteinase K; 5) 50 µl 0.5% Triton X-100, 6) 50 µl 0.5% Triton X-100 containing 100 µg/ml Proteinase K for 30 min at 37°C (Fig. S4). Protease inhibitors (complete Mini, Roche) were added to stop the digestion, samples were separated into supernatant and pellet, taken up in SDS-PAGE loading buffer (Invitrogen) and subjected to SDS-PAGE/Western Blot analysis. Sample loading was adjusted according to equivalent cell numbers. Blots were probed with mouse anti-HA antibodies (Roche; 1:1000), stripped (BioRad) and probed with anti-PTP2 (rabbit; 1:500).

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Fig. S1

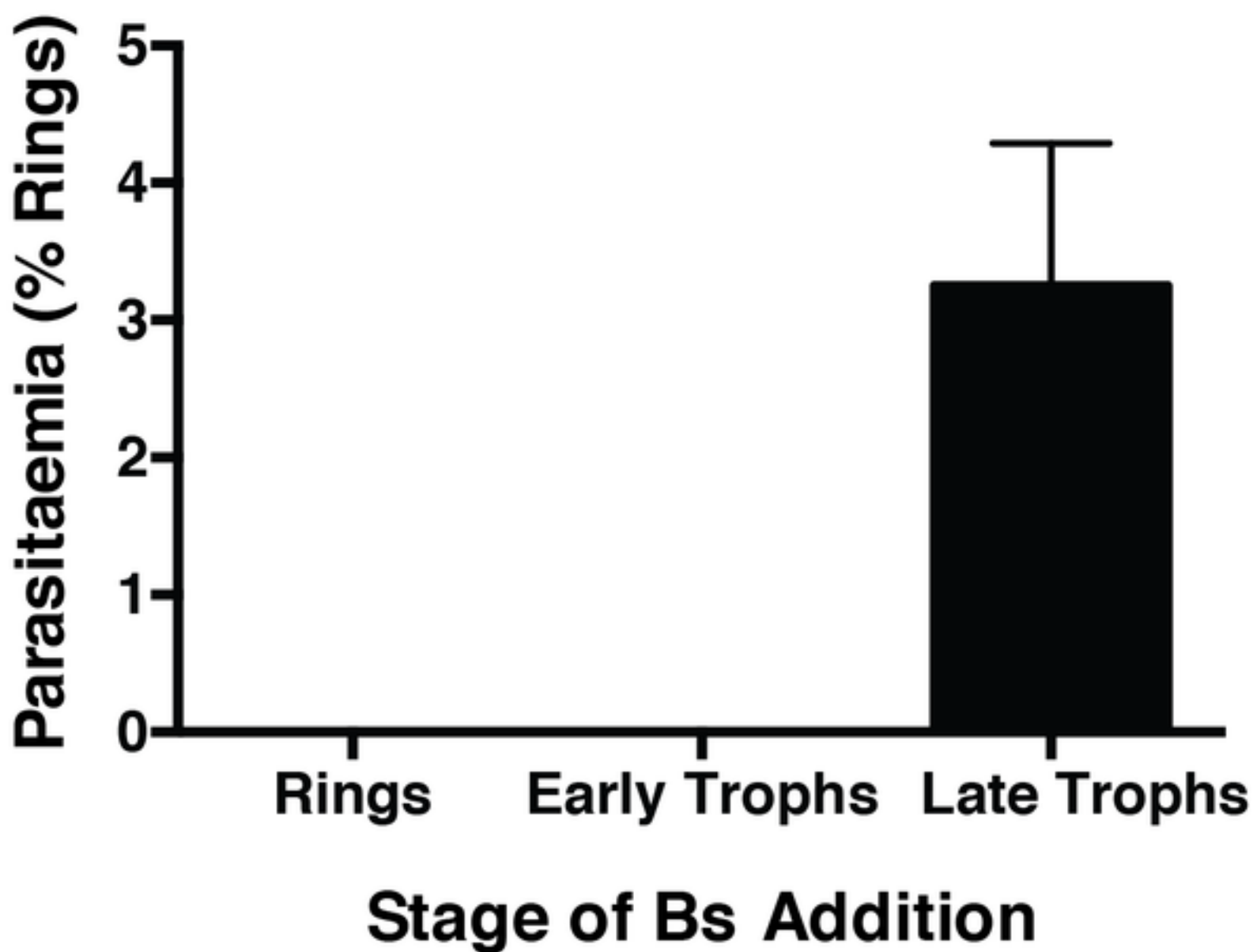


Fig. S2

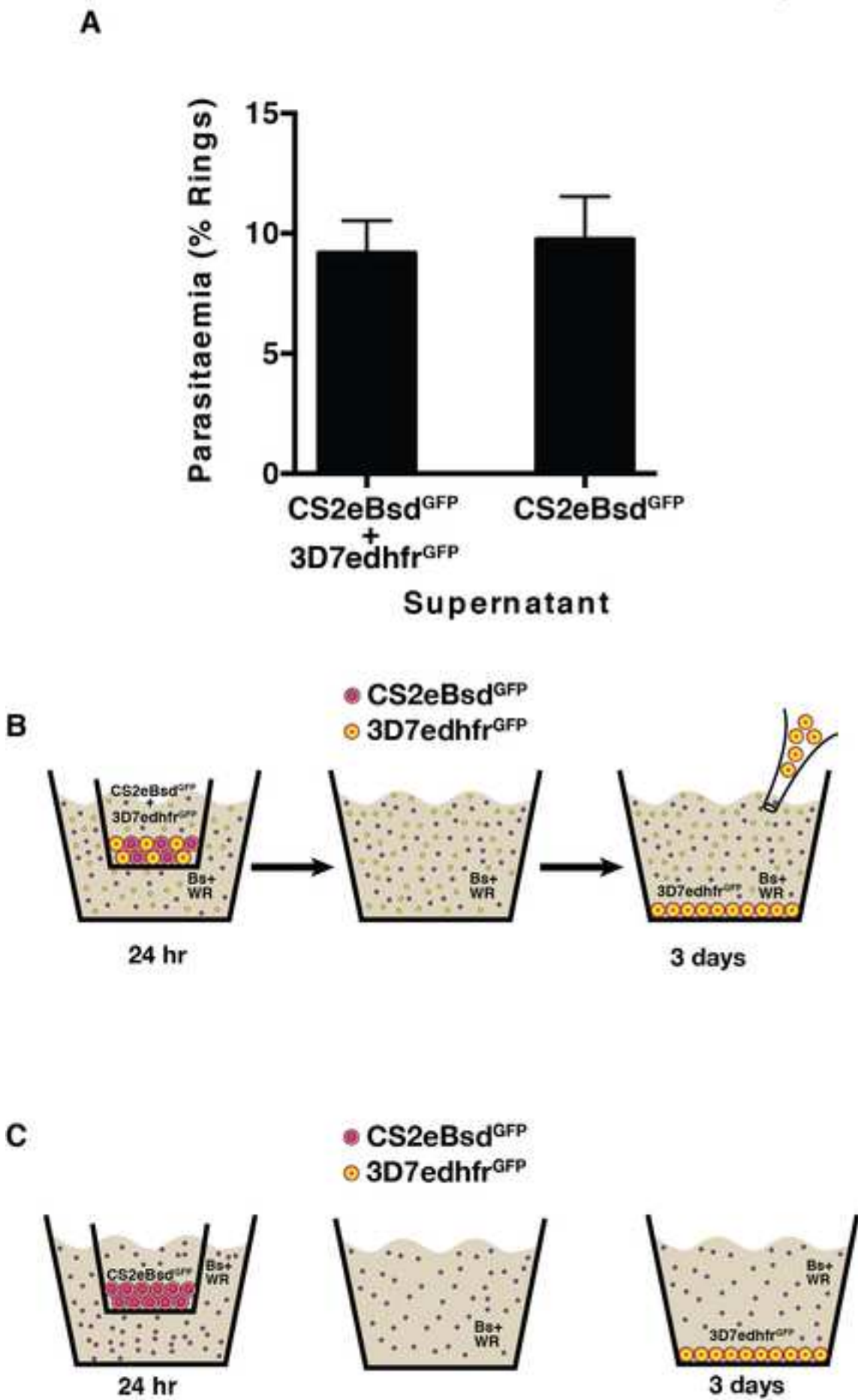


Fig. S3

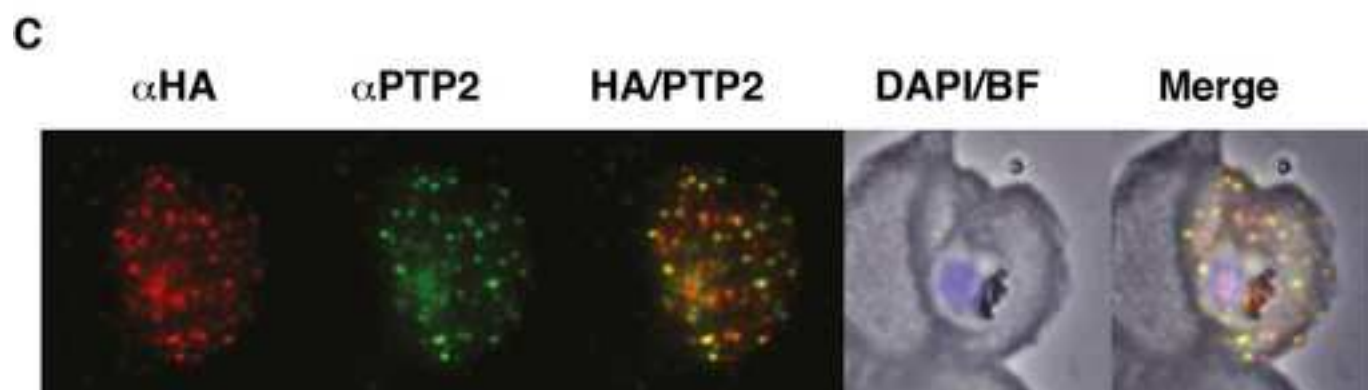
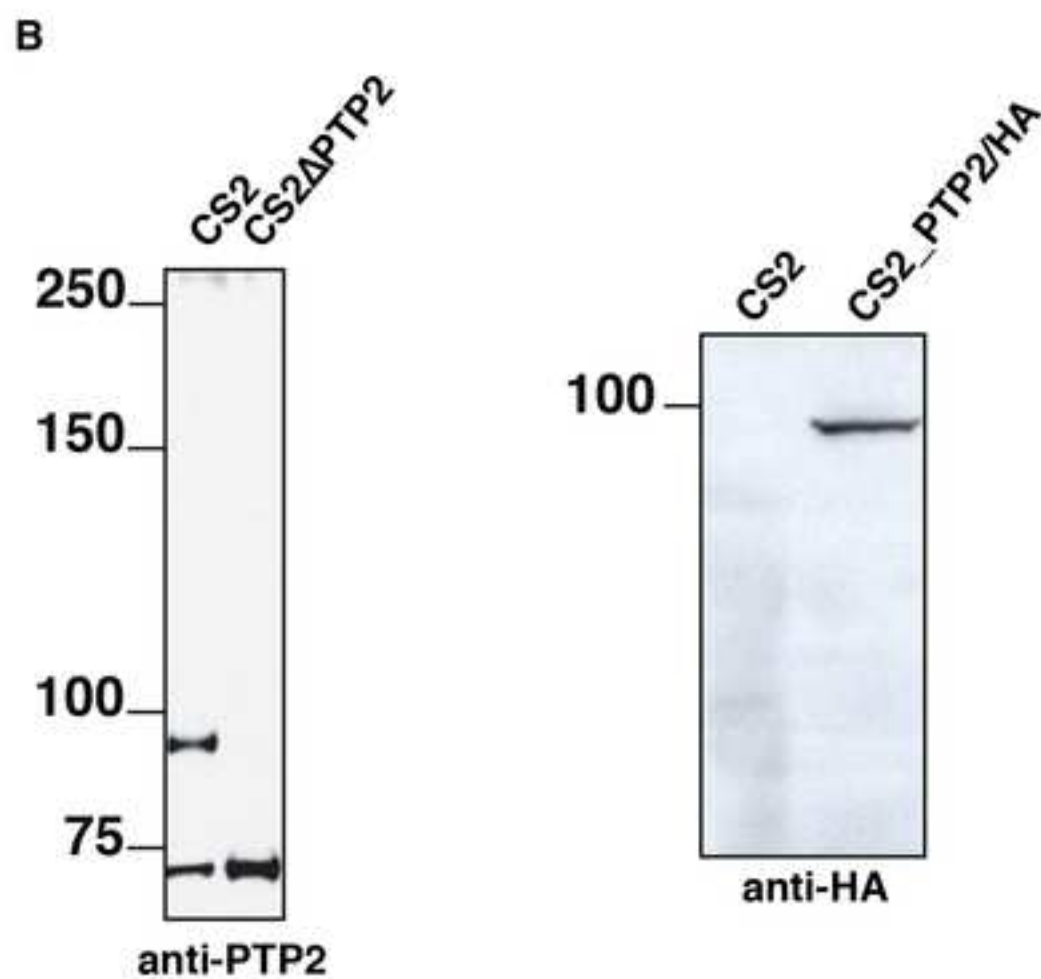
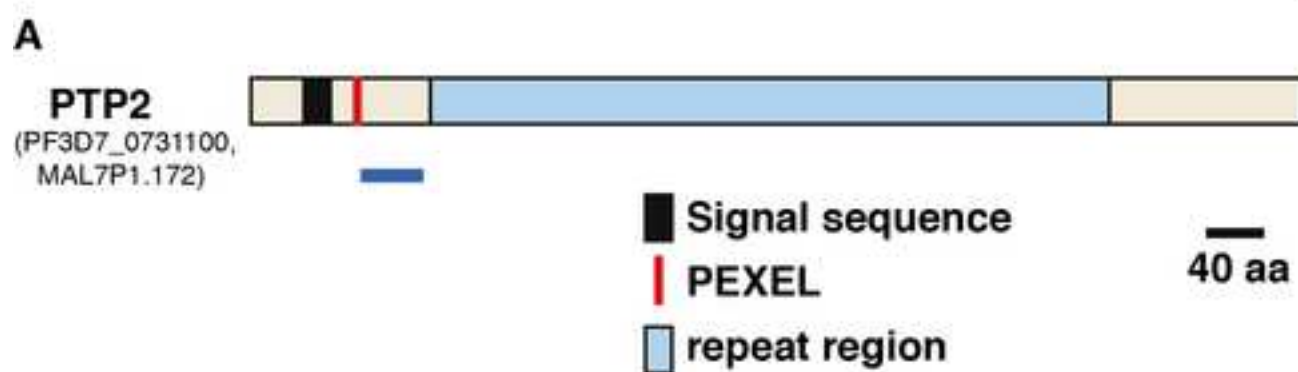
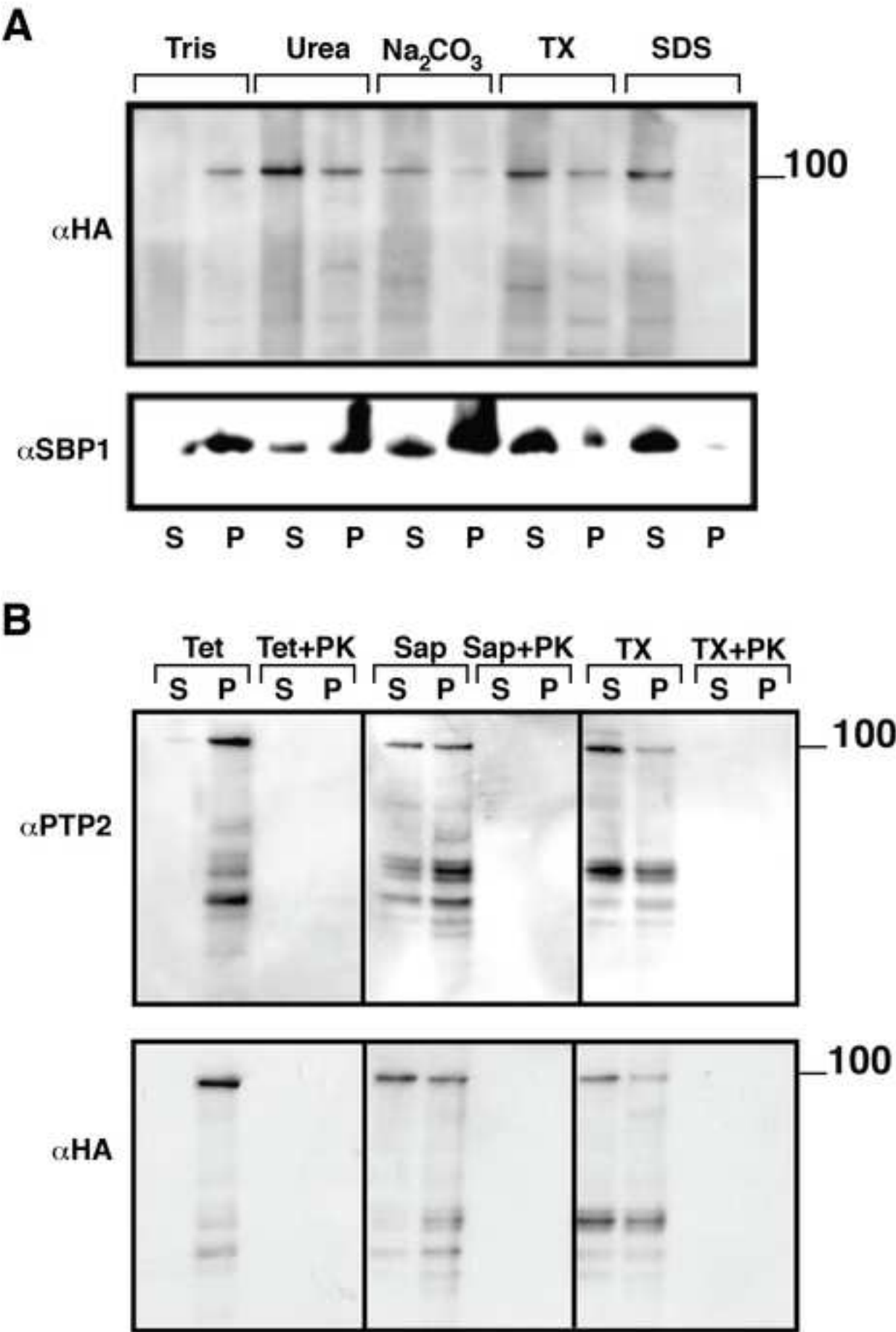


Fig. S4



ETOC Paragraph. 80 words

Cell-cell communication is a mechanism for information exchange promoting cell survival to control population density and differentiation. We show *Plasmodium falciparum*-infected red blood cells directly communicate within a population using exosome-like vesicles. These vesicles promote differentiation to sexual forms. We have also identified a *P. falciparum* protein required for efficient communication. This study reveals a previously unknown pathway of *P. falciparum* biology critical for survival in the host and transmission to mosquitoes.

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Author list: Neta Regev-Rudzki, Danny W Wilson, Teresa G Carvalho, Xavier Sisquella, Bradley M. Coleman, Melanie Rug, Dejan Bursac, Fiona Angrisano, Michelle Gee, Andrew F. Hill, Jake Baum and Alan F Cowman

Your name: Alan F. Cowman

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