Check for updates

RESEARCH ARTICLE

REVISED No evidence for Ago2 translocation from the host erythrocyte into the *Plasmodium* parasite [version 2; peer review: 2 approved]

Franziska Hentzschel [iD][1,2], Klara Obrova[2], Matthias Marti [iD][1]

[1]Wellcome Center for Integrative Parasitology; Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, G12 8TA, UK
[2]Center for Infectious Diseases, Parasitology Unit, Heidelberg University Hospital, Heidelberg, 69120, Germany

Abstract

Background: *Plasmodium* parasites rely on various host factors to grow and replicate within red blood cells (RBC). While many host proteins are known that mediate parasite adhesion and invasion, few examples of host enzymes co-opted by the parasite during intracellular development have been described. Recent studies suggested that the host protein Argonaute 2 (Ago2), which is involved in RNA interference, can translocate into the parasite and affect its development. Here, we investigated this hypothesis.
Methods: We used several different monoclonal antibodies to test for Ago2 localisation in the human malaria parasite, *P. falciparum* and rodent *P. berghei* parasites. In addition, we biochemically fractionated infected red blood cells to localize Ago2. We also quantified parasite growth and sexual commitment in the presence of the Ago2 inhibitor BCI-137.
Results: Ago2 localization by fluorescence microscopy produced inconclusive results across the three different antibodies, suggesting cross-reactivity with parasite targets. Biochemical separation of parasite and RBC cytoplasm detected Ago2 only in the RBC cytoplasm and not in the parasite. Inhibition of Ago2 using BCl-137 did not result in altered parasite development.
Conclusion: Ago2 localization in infected RBCs by microscopy is confounded by non-specific binding of antibodies. Complementary results using biochemical fractionation and Ago2 detection by western blot did not detect the protein in the parasite cytosol, and growth assays using a specific inhibitor demonstrated that its catalytical activity is not required for parasite development. We therefore conclude that previous data localising Ago2 to parasite ring stages are due to antibody cross reactivity, and that Ago2 is not required for intracellular *Plasmodium* development.

Open Peer Review

Reviewer Status ✓ ✓

	Invited Reviewers	
	1	**2**
version 2 (revision) 20 Nov 2020	✓ report	✓ report
	↑	↑
version 1 12 May 2020	? report	? report

1. **Julian Rayner** [iD], University of Cambridge, Cambridge, UK

2. **Tim W. Gilberger**, University of Hamburg, Hamburg, Germany
   Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany

Any reports and responses or comments on the article can be found at the end of the article.

This article is included in the Wellcome Centre for Integrative Parasitology gateway.

Corresponding author: Matthias Marti (Matthias.Marti@glasgow.ac.uk)

Author roles: Hentzschel F: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Obrova K**: Investigation; **Marti M**: Conceptualization, Funding Acquisition, Project Administration, Supervision, Writing – Review & Editing

Background

Malaria, caused by the protozoan parasite *Plasmodium*, remains a devastating disease affecting over 200 million people per year[1]. Clinical symptoms are initiated when parasites invade and replicate within red blood cells (RBCs). Several potent antimalarials are available, yet drug resistance is spreading as targeted *Plasmodium* proteins and pathways are amenable to mutation[2]. Host factors required for *Plasmodium* development could potentially offer novel drug targets that are less prone to resistance mutations[3,4]. However, while host receptors essential for parasite invasion into the RBC are intensively studied, little is known about host factors required for parasite growth and development within the RBC.

Studies by us and others have suggested possible involvement of the host protein Argonaute 2 (Ago2) in parasite development[5,6]. In many eukaryotic systems Ago proteins are core proteins of the RNA interference (RNAi) machinery. Directed by the complementary sequence of microRNAs (miRNAs), Ago proteins bind to target mRNAs and either suppress translation, or, in case of Ago2, the only catalytically active Ago protein in mammalians, directly cleave the mRNA target[7,8]. While mature RBCs lack a nucleus and thus *de novo* transcription, Ago2-mediated RNAi is important for RBC maturation from haematopoietic stem cells[9–11], i.e. erythropoiesis. Ago2 is essential for non-canonical processing and maturation of the miRNAs miR-451 and miR-486, major miRNAs involved in erythropoiesis[12,13]. Moreover, Ago2 and several miRNAs, including miR-451, are found in *P. falciparum*-infected and uninfected reticulocytes and mature RBCs[14–17]. We and others have found previously that infected RBCs (iRBCs) shed increased amounts of extravascular vesicles (EVs) compared to uninfected RBCs[5,6]. These iRBC-derived EVs contain Ago2-miRNA complexes that can alter endothelial cell function via RNAi, suggesting a contribution to vascular dysfunction

during malaria infection[5]. Intriguingly, we also localised host Ago2 to the parasite cytoplasm of ring-infected RBCs, suggesting possible Ago2 activity in the parasite[5,6]. *Plasmodium* parasites do not have an RNAi machinery including Ago proteins[18]. To enable manipulation of gene expression via RNAi, we have previously demonstrated successful expression of a human core RNAi machinery in the murine model *Plasmodium berghei* (*P. berghei*)[19]. Interestingly, we observed that the ectopic expression of Ago2 in *P. berghei* induced a minor growth defect in asexual parasite stages and downregulation of genes known to be targeted by post-transcriptional repression in female sexual stages (gametocytes). These observations show that Ago2 can interfere with parasite gene expression, supporting the hypothesis that host Ago2 is involved in *Plasmodium* blood stage development. Indeed, a recent study suggested that host Ago2 can downregulate virulence gene expression in *P. falciparum* as part of an innate resistance mechanism of the host RBC against the parasite[6].

In this work, we aimed to validate the localisation of host Ago2 to blood stage parasites and investigate if catalytically active host Ago2 is required for parasite development. Our data demonstrate that previously observed Ago2 localisation in the parasite by fluorescence microscopy is the result of antibody cross reactivity. Importantly, we detected no Ago2 in parasites when performing Western blotting on fractionated parasite samples, and inhibition of Ago2 catalytical activity did not impact asexual parasite growth and parasite development. We thus conclude that, at least *in vitro*, Ago2 is not a host factor important for intraerythrocytic parasite development.

Methods

Ethics statement

All animal experiments were performed according to European regulations concerning FELASA category B and GVSOLAS standard guidelines. Animal experiments were approved by German authorities (Regierungspraesidium Karlsruhe, Germany), § 8 Abs. 1 Tierschutzgesetz (TierSchG) under the license G-260/12 and were performed according to National and European regulations. Two female outbred NMRI mice (8- to 10-week-old) purchased from Janvier laboratories, France, were used. Mice were kept in groups of 2 to 4 mice per cage under specified pathogen-free (SPF) conditions within the animal facility at Heidelberg University (IBF) on a 12-hour light/dark cycle at 22°C (± 2°C) with *ad libitum* access to food and water.

P. falciparum in vitro culture

Parasite culture was performed as described previously[20]. *P. falciparum* parasites (strains 3D7, NF54 and 2004[20]) were kept in fresh type 0+ human erythrocytes (NHS National Services Scotland), suspended at 5% hematocrit in HEPES-buffered RPMI 1640 medium (Gibco™ 22400089) supplemented with 10% (w/v) heat inactivated human serum (Interstate Blood Bank), 0.05 mg/ml hypoxanthine (Gibco™) and 50 ng/ml gentamycin (Gibco™). To maintain the plasmid encoding the gametocyte reporter TdTomato, Pf2004 parasites were kept under 4 nM WR99210 (Jacobus Pharmaceuticals) selection pressure. Cultures were kept in a controlled environment at 37°C in a gassed chamber at 5% CO_2 and 1% O_2.

P. falciparum gametocyte commitment and mature gametocyte production

Parasite sexual commitment assay was performed as described previously[20]. Briefly, sorbitol-synchronised *P. falciparum* 2004 parasites expressing TdTomato under the gametocyte promoter etramp10.3 (PF10_0164/PF3D7_1016900)[20] were seeded at 24 hpi, 0.5% parasitemia into a 96-well plate adding varying BCI-137 (Merck-Millipore) concentrations (100 μM, 10 μM, 1 μM, 100 nM, 10 nM, 1 nM). For vehicle control, 1 % (v/v) and 0.1% DMSO (corresponding to 100 μM and 10 μM BCI-137, respectively), were added to parasites. Sexual commitment was induced by incubation in LysoPC-depleted minimal-fatty-acid medium for 24 h, while controls were maintained in complete medium. Medium was changed daily maintaining drug pressure. Parasitemia was assessed by SYBR green staining and flow cytometry on day 0, day 2 and day 4 post induction. On day 4, gametocytemia was determined as percentage of TdTomato-positive parasites. Parasite multiplication rate was calculated by dividing parasitemia on day 2 by starting parasitemia on day 0, sexual commitment was calculated by dividing gametocytemia on day 4 by parasitemia on day 2.

For production of mature gametocytes, sexual commitment of the *Pf*NF54 strain was induced as described above. After induction, asexual development was suppressed by addition of heparin to a final concentration of 0.23 mg/ml to the culture medium on days 2, 3 and 4 after induction. Mature gametocytes were obtained after 14 days of culture and fixed and stained for immunofluorescence assays (IFAs) as described below.

P. berghei infections

A female NMRI mouse was infected with *P. berghei* ANKA parasites by intraperitoneal injection of a cryostock containing approximately 1.5×10^7 infected RBCs in 100 μl blood and 200 μl freezing solution (10% glycerol in alsevier solution). Once parasitemia reached about 2–3%, the mouse was anaesthetised using an overdose of isoflurane and bled by cardiac puncture, and the blood processed for microscopy, as described below.

IFAs

IFAs were done as described previously[21]. Mixed-stage cultures of *Pf*3D7 parasites, sorbitol-synchronised *Pf*2004 parasites at 10, 22, 36, and 44 hours post invasion, mature *Pf*NF54 gametocytes or *P. berghei* ANKA parasites were collected and pelleted for 3 min at 1600 rpm. 50 μl RBC pellet were fixed in 1 ml 4% paraformaldehyde (PFA)/0.0075% glutaraldehyde for 20 min at 37°C. Cells were pelleted for 1 min at 3000 rpm and permeabilised in 1 ml 125 mM Glycine/0.1% Triton-X-100 in PBS for 10 min at RT. After pelleting and washing once with 3% BSA/PBS, cells were blocked overnight in 3% BSA/PBS. Primary antibodies were used at a dilution of 1:50 and incubated for 2 h at RT, shaking. RBCs were pelleted and washed 3 times for 10 min with 1% BSA/PBS. Secondary antibodies were diluted 1:300 in 3% BSA/PBS and incubated for 1 h at RT. RBCs were pelleted and washed 3 times for 10 min with PBS. The first washing step included Hoechst in a 1:3000 dilution to stain DNA. Primary and corresponding secondary antibodies used are depicted in Table 1.

For imaging, 2 μl of stained parasite pellet were placed onto a glass slide and covered with a coverslip. Imaging was performed on a confocal spinning disc microscope (Nikon) using a 100x objective or a widefield DMi8 microscope (Leica) using a 60x objective and images were processed with FIJI/ImageJ (v 2.0.0-rc-69/1.52p)[22,23].

Table 1. Antibodies used in this study.

Primary Antibody	Secondary antibody (IFA)	Secondary Antibody (WB)
Rabbit-αAgo2 (EPR10411), Abcam Cat# ab186733, RRID:AB_2713978	Goat-αRabbit, Alexa Fluor 488, Thermo Fisher Scientific Cat# A-11008, RRID: AB_143165	IRDye® 680RD Donkey anti-Rabbit IgG Secondary Antibody, LI-COR Biosciences Cat# 926-68073, RRID:AB_10954442
Mouse-αAgo2 (2E12-1C9), Abcam Cat# ab57113, RRID:AB_2230916	Goat-αMouse, Alexa Fluor 488, Thermo Fisher Scientific Cat# A-11001, RRID: AB_2534069 Goat-αMouse, Alexa Fluor 594, Thermo Fisher Scientific Cat# A-11032, RRID: AB_2534091	IRDye® 680RD Donkey anti-Mouse IgG Secondary Antibody, LI-COR Biosciences Cat# 926-68072, RRID:AB_10953628
Rat-αAgo2 (11A9), Thermo Fisher Scientific Cat# 14-6519-82, RRID:AB_2784637	Goat-αRat, Alexa Fluor 488, Thermo Fisher Scientific Cat# A-11006, RRID:AB_2534074	-
Mouse-α *Pb*Hsp70, RRID:AB_2650482[24]	Goat-αMouse, Alexa Fluor 594, Thermo Fisher Scientific Cat# A-11032, RRID: AB_2534091	-
Mouse α-αTubulin, Sigma-Aldrich Cat# T5168, RRID:AB_477579	Goat-αMouse, Alexa Fluor 594, Thermo Fisher Scientific Cat# A-11032, RRID: AB_2534091	-
Rabbit-α*Pf*BIP (antibody provided by MR4; clone MRA19[25], RRID:AB_2716735)	-	IRDye® 800CW Donkey anti-Rabbit IgG Secondary Antibody, LI-COR Biosciences Cat# 926-32213, RRID:AB_621848
Mouse-αHsHsp70, Santa Cruz Biotechnology Cat# sc-24, RRID:AB_627760	-	IRDye® 800CW Donkey anti-Mouse IgG Secondary Antibody, LI-COR Biosciences Cat# 926-32212, RRID:AB_621847

IFA: Immunofluorescence assay, WB: Western blot.

Parasite fractionation and western blotting

*P. falciparum Pf*2004 or 3D7 cultures were sorbitol-synchronised and harvested at 10, 22, 36 and 44 hpi and a parasitemia of 5% by pelleting for 3 min at 1600 rpm. For the total sample, 1×10^9 RBCs were washed in 1 ml PBS and resuspended to a final volume of 500 µl parasite lysis buffer supplemented with 1x protease inhibitor cocktail (PIC). For fractionation, 2×10^9 RBCs were washed in 1 ml PBS and lysed for 5 min on ice in 1 ml final volume of 0.015 % saponin/PBS (w/v). After 3 min centrifugation at full speed, the supernatant was transferred to a new tube and supplemented with 1x PIC. The pellet was washed 3 times in 1 ml PBS and resuspended in 50 µl parasite lysis buffer supplemented with 1x PIC.

For western blotting, samples were denatured in 1x SDS loading dye/0.05 M DTT for 5 min at 95°C and 4×10^6 RBCs (total and supernatant) or 2×10^7 iRBC (pellet) per lane were separated on a 4–12% Bis-Tris protein gel (NuPage™) and transferred onto Amersham Hybond LFP 0.2 PVDF Western blotting membranes (GE Life Sciences). Blots were blocked for 1 h in 1% fish gelatin/TBS and primary antibodies (Table 1, dilution 1:300) incubated over night at 4 °C. Secondary antibodies (Table 1) were diluted 1:20000 in 1% fish gelatin/0.01% SDS/TBS-T and incubated for 1 h at RT. Blots were developed on a Licor Odyssey® CLx. Images of the whole blots are depicted in *Extended data*, Extended Figure 1 and 2[26]. Raw western blot images are available as *Underlying data*[27].

Results

Ambiguous Ago localization in parasite and host cell by fluorescence microscopy

In a first step, we aimed to verify the previously published results of Ago2 localisation to *P. falciparum* ring stages. To this end, we performed IFAs on mixed *P. falciparum* 3D7 and

P. berghei ANKA blood stages using the commercial Ago2 antibody EPR10411, which binds to a central peptide epitope of human and murine Ago2. We observed a clear punctate pattern of host Ago2 in the cytoplasm of all asexual stages of *P. falciparum*, as well as *P. berghei* rings and, to a lesser extent, trophozoites (Figure 1A, B).

As this localisation is in contrast to the previous reports which detected Ago2 only in ring stage parasites, we repeated the experiment with two additional commercial monoclonal antibodies (mAbs): 2E12-1C9, which has previously been used[5,6], and 11A9, which has not been tested with *Plasmodium* before. The clone 2E12-1C9 was raised against the Ago2 C-terminus (target epitope unknown), while clone 11A9 was raised against a N-terminal peptide. The respective Ago2 target sites of the different antibodies are depicted in Figure 2A. All three Ago2 antibodies detected Ago2 as punctate structures in the RBC cytoplasm and close to the membrane, matching the previously published localisation of Ago2 in uninfected RBC ghosts[17]. However, the antibodies showed only minor overlap in localization, suggesting some non-specific reactivity (Figure 2B).

To investigate the localization of these antibodies in iRBCs, we used synchronised *Pf*2004 parasites at 10, 22, 36 and 48 hpi, as well as mature gametocytes. Again, only minor overlap in parasite staining was observed across the different antibodies used (Figure 2C). While EPR10411 labelled all asexual stages (see also Figure 1A) and a fraction of mature *P. falciparum* gametocytes, 2E12-1C9 only stained 10 hpi ring stages, replicating the previously published data with this antibody[5,6]. In contrast, 11A9 did not localise to any stage of the parasites (Figure 2D). In conclusion, three different antibodies directed against Ago2 resulted in three different staining
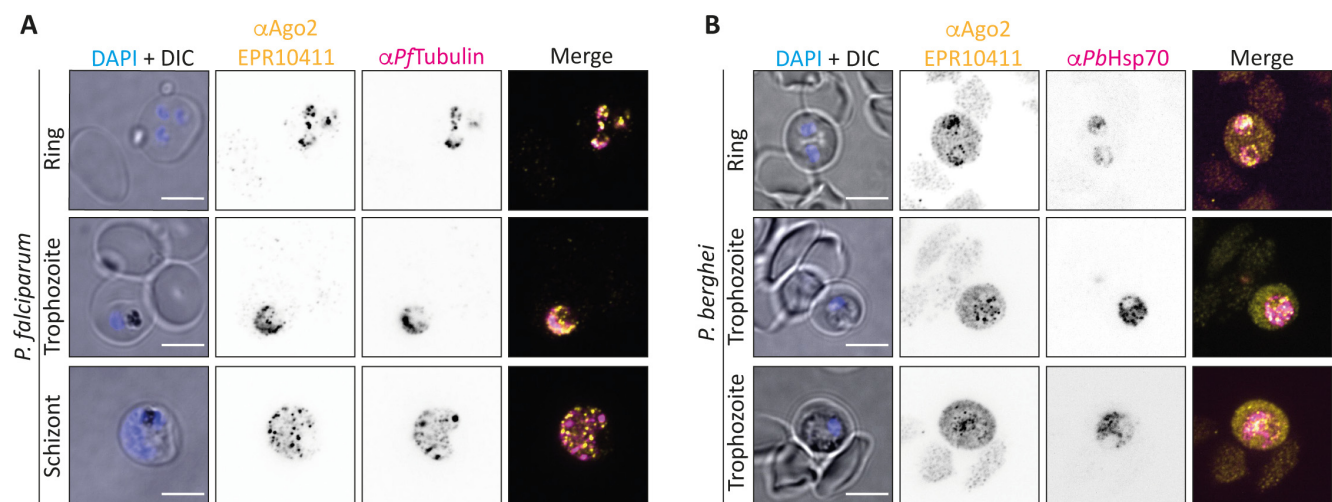
Figure 1. Localisation of Ago2 to the iRBC. (A) IFA of *P. falciparum* 3D7 blood stages. Yellow: Ago2 (EPR10411), magenta: *Pf*Tubulin. (**B**) IF staining of *P. berghei* ANKA rings and trophozoites. Yellow: Ago2 (EPR10411), magenta: *Pb*Hsp70. Nuclei were stained with Hoechst (blue). Images were taken on a Nikon spinning disc confocal microscope (100x objective). Representative images of at least 5 per condition are shown. Controls using only a secondary antibody revealed no unspecific staining. Scale bar indicates 5 µm.
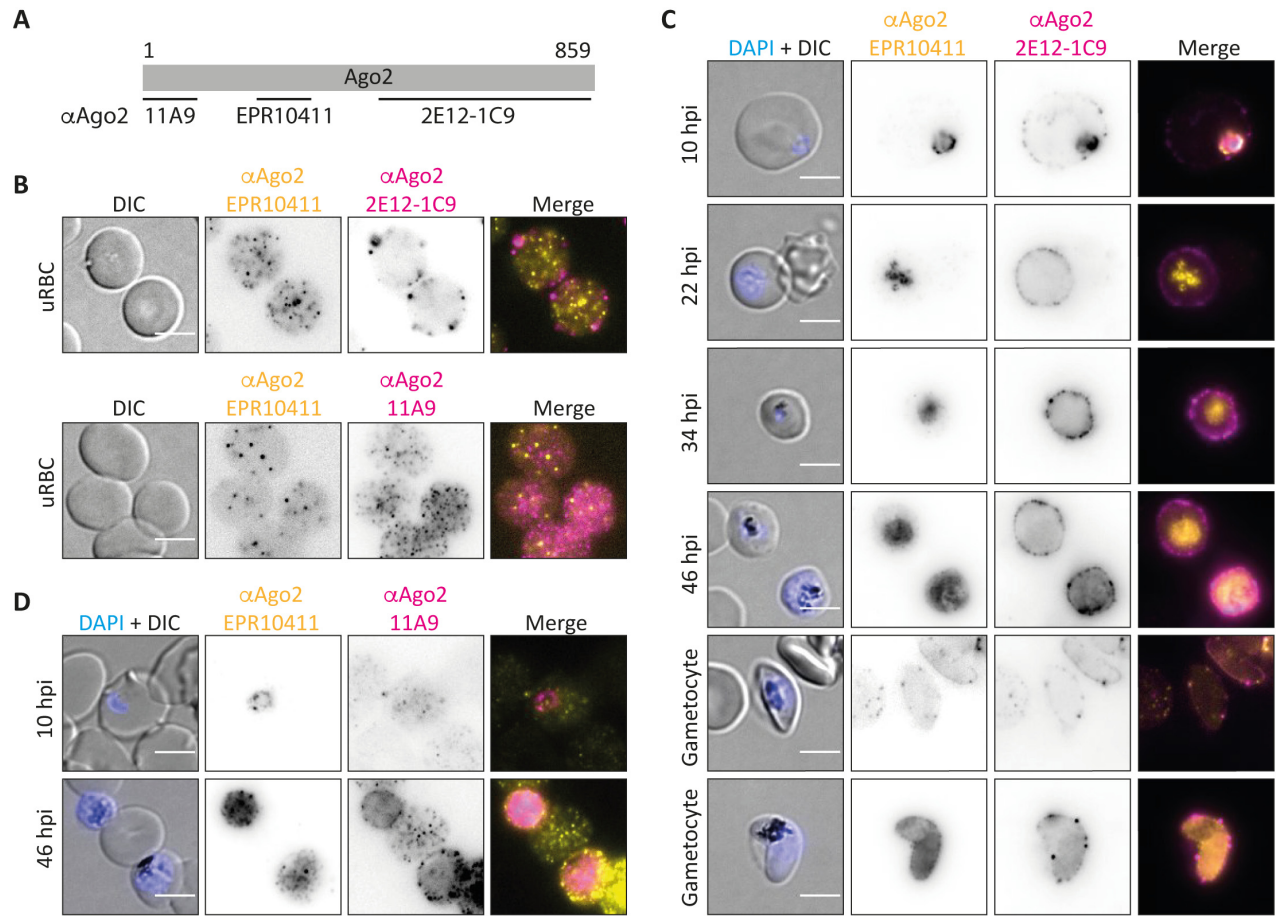
Figure 2. Further analysis of Ago2 localization to the iRBC. (**A**) Binding sites of αAgo2 antibodies used. The monoclonal antibodies 11A9 and EPR10411 were raised against an N-terminal and a centrally located peptide epitope, respectively. The antibody 2E12-1C9 is a monoclonal antibody raised against the whole C-terminus of hAgo2. (**B**) IFA of uninfected RBCs. Yellow: Ago2 (EPR10411), magenta: Ago2 (either 2E12-1C9 (top) or 11A9 (bottom)). (**C**) IFA of *P. falciparum* 2004 parasites at 10, 22, 34 and 46 hours post invasion (hpi) and mature *Pf*NF54 gametocytes. Yellow: Ago2 (EPR10411), magenta: Ago2 (2E12-1C9). Contrast and brightness of EPR10411 panels were individually adjusted to optimise image quality, thus, signal strength cannot be compared between panels. (**D**) IFA of *P. falciparum* 2004 parasites at 10 and 46 hpi. Yellow: Ago2 (EPR10411), magenta: Ago2 (11A9). Nuclei were stained with Hoechst (blue). Images were taken on a Leica widefield microscope (60x objective). Representative images of at least 5 per condition are shown. Controls using only a secondary antibody revealed no unspecific staining. Scale bar indicates 5 μm.

patterns in IFAs strongly suggesting that at least part of the signal is non-specific. Raw images used to generate Figure 1 and Figure 2 are available as *Underlying data*[27].

No evidence for Ago2 localization in the parasite by biochemical fractionation

To independently investigate Ago2 localisation in parasite and host cell, we performed fractionation experiments followed by western blotting. Synchronised *P. falciparum Pf*2004 or *Pf3D7* parasites at 10, 22, 36 and 44 hpi were lysed using saponin and separated into a supernatant fraction containing the RBC cytoplasm and parasitophorous vacuole (PV) content, and the pellet containing membranes and parasite cytoplasm. Western blots of total, supernatant and pellet samples were probed for

Ago2 using the antibodies 2E12-1C9 and EPR10411, which both detected Ago2 signal in parasites by IFA (Figure 1A, Figure 2C). As controls for the fractionation protocol we used antibodies against the RBC cytosol protein human heat shock protein 70 (hHsp70) and the parasite ER protein *Pf*BIP. In non-fractionated (total) samples, we detected bands corresponding to Ago2, hHsp70 and *Pf*BIP (Figure 3A, B, left panels). As expected, hHsp70 was only detected in the RBC cytosol (RBC, middle panels), while *Pf*BIP was only detected in the parasite (right panels), indicating clean separation of these two fractions. Intriguingly, both antibodies against Ago2 detected a band corresponding to the expected size of Ago2 only in the supernatant fraction (RBC cytosol), independent of parasite stage. No other bands were detected across the whole blots (*Extended*
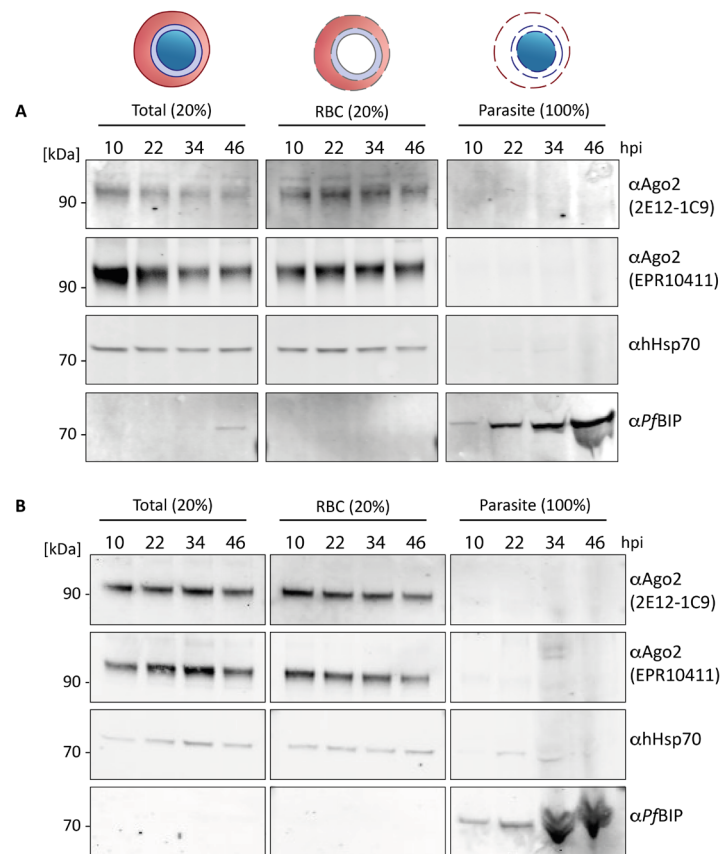
Figure 3. Western blot of fractionated *P. falciparum*-infected iRBCs. A) *Pf*2004 or **B**) *Pf*3D7 iRBCs at 10, 22, 34 and 46 hours post invasion (hpi) were separated via saponin lysis into erythrocyte cytoplasm and PV content (RBC) and parasite cytoplasm and membranes (parasite). Western blot analysis was performed on total protein (left panel, 20 % loaded), saponin supernatant containing RBC and PV cytoplasm (middle panel, 20 % loaded) and pellet containing parasite cytoplasm (right panel, 100 % loaded). Antibodies targeting the RBC housekeeper hHsp70 (expected size human Hsp70: 70 kDa) and the parasite housekeeper *Pf*BIP served as control for proper fractionation. Using two Ago2 antibodies, Ago2 was only detected in the total and the RBC fraction (expected size Ago2 95 kDa).

data, Extended Figures 1, 2A, B)[26]. This finding contradicts our IFA data (Figure 1, Figure 2) and suggests that Ago2 is not localized to the parasite.

Inhibition of Ago2 function does not affect parasite growth

To directly investigate whether host Ago2 plays a role in blood stage malaria parasites, we tested the effect of an Ago2 inhibitor against *P. falciparum* parasites. BCI-137 is a cell permeable Ago2 inhibitor that prevents miRNA-binding and thus functionally inhibits Ago2[28]. First, we tested the effect of BCl-137 on parasite growth using a dilution series of the compound. As a previous study inhibiting the host protein acylpeptide hydrolase only observed an inhibitory effect after two asexual replication cycles[29], we quantified parasite multiplication rate (PMR) for two consecutive cycles but observed no change in PMR compared to vehicle (DMSO) up to 100 μM of compound (Figure 4A). We also tested the effect on gametocyte production using our previously published assay to induce sexual commitment via

depletion of LysoPC[20], and again no phenotype was observed (Figure 4B). A higher concentration of 1 mM BCI-137 led to a decreased PMR and increased sexual commitment, yet this was due to toxic concentrations of the solvent DMSO (*Extended data*, Extended Figure 3)[26]. Flow cytometry output files are available as *Underlying data*[27]. Altogether these data demonstrate that inhibition of Ago2 function with BCI-137 does not affect parasite multiplication rate or gametocyte production, even at micromolar concentrations.

Discussion

In this study we have followed up previous reports describing the unusual localisation of a host protein, Ago2, in the intracellular blood stages of *Plasmodium* parasites. Malaria parasites take up nutrients from the host cell environment through non-selective pores in the parasitophorous vacuolar membrane (PVM)[30,31]. Large scale uptake of host cytosolic material into the parasite, including haemoglobin and other essential nutrients, is additionally facilitated by a recently characterized
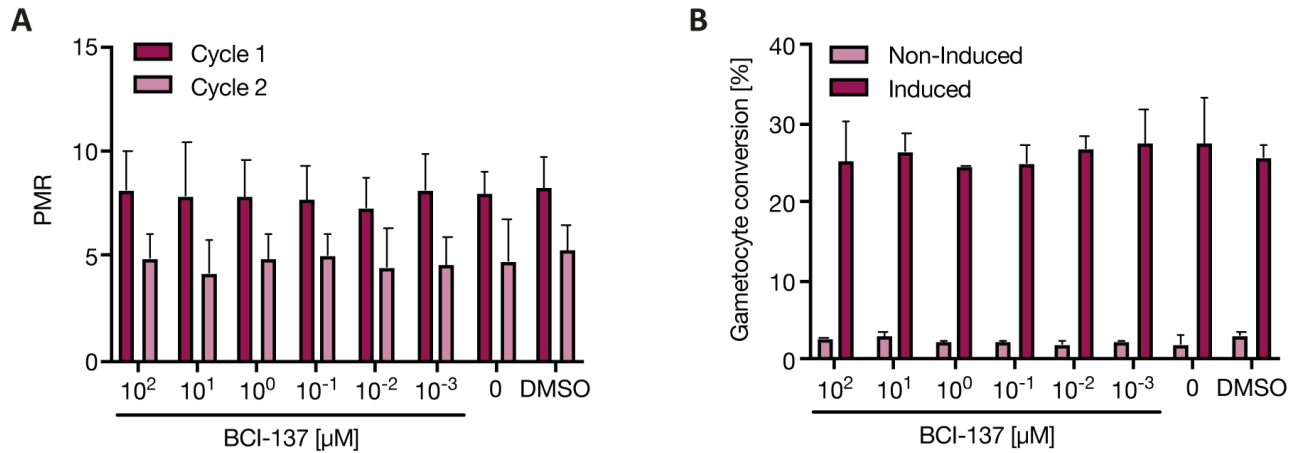
Figure 4. Parasite growth and commitment in presence of an inhibitor of hAgo2. *Pf*2004 parasites were treated with the Ago2 inhibitor BCI-137 at various concentrations or vehicle (DMSO) and assessed for asexual parasitemia and gametocytemia by flow cytometry. **A**) Parasite multiplication rate (PMR) over two cycles of asexual replication. **B**) Gametocyte conversion rate under non-induced and gametocyte-induced conditions. Note: no RBC control is included as it is not possible to directly measure Ago2 activity in RBCs due to their lack of transcriptional activity. However, we used the inhibitor at a tenfold higher maximal concentration compared to what has been shown to be sufficient to inhibit Ago2-miRNA binding[28]. Shown is the mean ± SD of n = 2, with 3 technical replicates each.

endocytosis pathway[32]. Parasites also take up host enzymes and co-opt their function, as demonstrated by the import of human peroxiredoxin 2 for peroxide detoxification[33] or of erythrocyte acylpeptide hydrolase[29].

The reported localisation of Ago2 in early ring stage parasites and the observed phenotype upon Ago2 over-expression prompted us to investigate if indeed host Ago2 function had also been co-opted by the malaria parasite. We used three independent approaches to test for a possible role of Ago2 in parasite development. First, we localised Ago2 in iRBCs using a series of monoclonal antibodies that provided ambiguous results. Second, we fractionated iRBCs into host cytoplasm and parasite and performed Western blotting with two antibodies targeting Ago2, demonstrating Ago2 in the RBC fraction only. Third, we measured parasite growth and sexual commitment upon incubation with the Ago2 inhibitor BCI-137, resulting in no discernible phenotype.

In nucleated cells, Ago2 is known to localise both to the cytoplasm and to cytoplasmic ribonucleoprotein granules, e.g. GW/P bodies or stress granules, which are sites of miRNA-mediated mRNA degradation[34,35]. To our knowledge, it has not yet been investigated if such granules are present in erythrocytes and the subcellular localisation of Ago2 in RBCs is unknown, with the exception of one study which localised Ago2 to punctuate structures close to the membrane of erythrocyte ghosts; no localisation in the RBC cytoplasm was performed[17]. In the present work, we observed a speckled localisation for Ago2 within the erythrocyte, resembling the punctuate localisation of Ago2 to P bodies in nucleated cells[34]. However, in our hands, none of the three different commercial antibodies against Ago2 showed substantial co-localisation in IFA studies. Antibody 2E12-1C9

is known to cross-react with Ago1, Ago3 and Ago4, yet erythroid cells almost exclusively express Ago2[12]. Ago2 is subject to a variety of posttranslational modifications, some of which can alter the subcellular localisation of the protein[36]. For example, phosphorylation of Ago2 at position Ser387 increases its localisation to P bodies[37]. Intriguingly, the epitope for the antibody EPR10411 encompasses Ser387, and phosphorylation at this site might affect binding of the antibody to Ago2. Other post-translational modifications might exist at the epitopes for the other antibodies, and it is thus tempting to speculate that the different antibodies recognise different subpopulations of post-translationally modified Ago2 in the RBC.

Using three different antibodies, we also observed ambiguous Ago2 staining in the parasite, with the antibody 11A9 not localizing to the parasite at all, the antibody 2E12-1C12 localizing only to ring stages, and the antibody EPR10411 to all asexual parasites and some gametocytes. Importantly, we did not detect Ago2 in the parasite fraction at any time point of the asexual cycle by western blot using two different antibodies, arguing against the presence of Ago2 in the parasite cytoplasm. We previously detected a weak band in the parasite fraction of trophozoites with one of these antibodies[5]. This may have been the result of incomplete separation of the fractions, as in absence of an antibody targeting a cytoplasmic RBC marker protein, a contamination of the parasite fraction with host cytosol protein cannot be excluded. Similarly, a western blot in another study demonstrated Ago2 in the parasite fraction, but lacked appropriate controls to demonstrate clean fractionation[6].

In absence of a *Plasmodium* Ago2 homologue, it remains unknown which parasite protein is detected by αAgo2 antibodies in IFAs. A BLAST search with the sequence of the linear peptide

epitope of the antibody EPR10411 (as obtained from the supplier) against a Pf database did not yield any meaningful match. In addition, Western blotting of parasite fractions did not yield any clean band across the whole blot (*Extended data*, Extended Figures 1,2)[26], indicating that the observed Ago2 localization in the parasite with both antibodies using IFA is the result of cross reactivity with a conformational parasite epitope. In IFAs, EPR10411 produced a specific and strong signal across all parasite stages which increased during asexual development, while 2C12-E2 was only detected in early ring stages, suggesting that the two antibodies cross-react with different parasite antigens.

We also did not observe any inhibitory effect using the membrane-permeable drug BCI-137 on parasite development. This finding is in line with previous observations that ectopic expression of Ago2-dependent miRNAs in *P. berghei* does not regulate gene expression in absence of ectopically expressed Ago2[19]. Altogether our data do not support the hypothesis that Ago2 is transferred from host to parasite, and/or that it performs a relevant role in parasite development.

Our study emphasises the importance of using proper controls and orthogonal methods to corroborate initial findings. Notably, the Ago2 antibody 11A9 has been previously reported to cross-react with the nuclear protein SMARCC1 in chromatin immunoprecipitation (IP) studies[38]. Given that we were not able to detect Ago2 in *Plasmodium*, we presume that previous results of an Ago2-IP from parasites were also influenced by cross reactivity[6]. In summary, we conclude that there is no Ago2-mediated miRNA activity in malaria parasites, despite a previous report suggesting otherwise.

Data availability
Underlying data
Figshare: No evidence for Ago2 translocation from the host erythrocyte to the Plasmodium parasite. https://doi.org/10.6084/m9.figshare.c.4960649[27].

This project contains the following underlying data:
- Figure 1B - PbANKA + EPR10411 - Raw Image Files (TIF; https://doi.org/10.6084/m9.figshare.12229037).
- Figure 1B - Pf3D7 + EPR10411 - Raw Image Files (TIF; https://doi.org/10.6084/m9.figshare.12229050).
- Figure 2D - Figure 2D - EPR10411 + 2E12-1C9 - Raw Image Files (TIF; https://doi.org/10.6084/m9.figshare.12229107).
- Figure 3 - Western Blots Raw Data (TIF; https://doi.org/10.6084/m9.figshare.12229169).
- Figure 4, Extended Figure 1 - Flow Cytometry Raw Data (XLSX; https://doi.org/10.6084/m9.figshare.12229160).

Extended data
Figshare: Extended Figures 1 and 2. https://doi.org/10.6084/m9.figshare.12229028[26].

This project contains the following extended data:
- **Extended Figure 1 (PDF). Whole blots of Western blot of fractionated P. falciparum-infected iRBCs (strain Pf2004).** iRBCs at 10, 22, 34 and 46 hours post invasion (hpi) were separated via saponin lysis into into erythrocyte cytoplasm and PV content (RBC) and parasite cytoplasm (parasite). Western blot analysis was performed on total protein (total, 20 % loaded), saponin supernatant containing RBC and PV cytoplasm (RBC, 20 % loaded) and pellet containing parasite cytoplasm (parasite, 100 % loaded). (A) Antibody αAgo2 2E12-1C9 (expected size Ago2 95 kDa). (B) Antibody αAgo2 EPR10411 (expected size Ago2 95 kDa). (C) Antibody αhHsp70 (expected size human Hsp70: 70 kDa). (D) Antibody αPfBIP (expected size 72 kDa). kDa: kilo Dalton, hpi: hours post invasion, M: Marker Li-Cor Chamaeleon DUO.
- **Extended Figure 2 (PDF). Whole Western blots of fractionated *P. falciparum*-infected iRBCs (strain *Pf*3D7).** iRBCs at 10, 22, 34 and 46 hours post invasion (hpi) were separated via saponin lysis into into erythrocyte cytoplasm and PV content (RBC) and parasite cytoplasm (parasite). Western blot analysis was performed on total protein (total, 20 % loaded), saponin supernatant containing RBC and PV cytoplasm (RBC, 20 % loaded) and pellet containing parasite cytoplasm (parasite, 100 % loaded). (A) Antibody αAgo2 2E12-1C9 (expected size Ago2 95 kDa). (B) Antibody αAgo2 EPR10411 (expected size Ago2 95 kDa). (C) Antibody αhHsp70 (expected size human Hsp70: 70 kDa). (D) Antibody αPfBIP (expected size 72 kDa). kDa: kilo Dalton, hpi: hours post invasion, M: Marker Li-Cor Chamaeleon DUO.
- **Extended Figure 3 (PDF). Parasite growth and commitment in presence of an inhibitor of hAgo2.** Pf2004 parasites were treated with the Ago2 inhibitor BCI-137 at various concentrations or vehicle (DMSO) and assessed for asexual parasitemia and gametocytemia by flow cytometry. (A) Parasite multiplication rate (PMR) over two cycles of asexual replication. (B) Gametocyte conversion rate under non-induced and gametocyte-induced conditions. DMSO (1%) is the vehicle control corresponding to 100 μM BCI-137, 0.1 % DMSO 1000 is the vehicle control corresponding to 10 μM BCI-137. Shown is the mean ± SD of n = 2, with 3 technical replicates each.

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

References

1. World Health Organization: **World Malaria Report 2019**. 2019.
   Reference Source

2. Blasco B, Leroy D, Fidock DA: **Antimalarial drug resistance: Linking** *Plasmodium falciparum* **parasite biology to the clinic.** *Nat Med.* 2017; **23**(8): 917–928.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

3. Langhorne J, Duffy PE: **Expanding the antimalarial toolkit: Targeting host-parasite interactions.** *J Exp Med.* 2016; **213**(2): 143–153.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

4. Glennon EKK, Dankwa S, Smith JD, *et al.*: **Opportunities for Host-targeted Therapies for Malaria.** *Trends Parasitol.* 2018; **34**(10): 843–860.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

5. Mantel PY, Hjelmqvist D, Walch M, *et al.*: **Infected erythrocyte-derived extracellular vesicles alter vascular function via regulatory Ago2-miRNA complexes in malaria.** *Nat Commun.* 2016; **7**: 12727.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

6. Wang Z, Xi J, Hao X, *et al.*: **Red blood cells release microparticles containing human argonaute 2 and miRNAs to target genes of** *Plasmodium falciparum*. *Emerg Microbes Infect.* 2017; **6**(8): e75.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

7. Meister G: **Argonaute proteins: functional insights and emerging roles.** *Nat Rev Genet.* 2013; **14**(7): 447–459.
   PubMed Abstract | **Publisher Full Text**

8. Jonas S, Izaurralde E: **Towards a molecular understanding of microRNA-mediated gene silencing.** *Nat Rev Genet.* 2015; **16**(7): 421–433.
   PubMed Abstract | **Publisher Full Text**

9. Cheloufi S, Dos Santos CO, Chong MM, *et al.*: **A dicer-independent miRNA biogenesis pathway that requires Ago catalysis.** *Nature.* 2010; **465**(7298): 584–589.
   PubMed Abstract | **Publisher Full Text** | **Free Full Text**

10. O'Carroll D, Mecklenbrauker I, Das PP, *et al.*: **A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway.** *Genes Dev.* 2007; **21**(16): 1999–2004.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

11. Listowski MA, Heger E, Bogusławska DM, *et al.*: **MicroRNAs: Fine tuning of erythropoiesis.** *Cell Mol Biol Lett.* 2013; **18**(1): 34–46.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

12. Jee D, Yang JS, Park SM, *et al.*: **Dual Strategies for Argonaute2-Mediated Biogenesis of Erythroid miRNAs Underlie Conserved Requirements for Slicing in Mammals Article Dual Strategies for Argonaute2-Mediated Biogenesis of Erythroid miRNAs Underlie Conserved Requirements for Slicing in Mam.** *Mol Cell.* 2018; **69**(2): 265–278.e6.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

13. Yang JS, Lai EC: **Dicer-independent, Ago2-mediated microRNA biogenesis in vertebrates.** *Cell Cycle.* 2010; **9**(22): 4455–4460.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

14. Azzouzi I, Moest H, Wollscheid B, *et al.*: **Deep sequencing and proteomic analysis of the microRNA-induced silencing complex in human red blood cells.** *Exp Hematol.* 2015; **43**(5): 382–392.
    PubMed Abstract | **Publisher Full Text**

15. Rathjen T, Nicol C, McConkey G, *et al.*: **Analysis of short RNAs in the malaria parasite and its red blood cell host.** *FEBS Lett.* 2006; **580**(22): 5185–5188.
    PubMed Abstract | **Publisher Full Text**

16. Xue X, Zhang Q, Huang Y, *et al.*: **No miRNA were found in Plasmodium and the ones identified in erythrocytes could not be correlated with infection.** *Malar J.* 2008; **7**: 47.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

17. Basu A, Harper S, Pesciotta EN, *et al.*: **Proteome analysis of the triton-insoluble erythrocyte membrane skeleton.** *J Proteomics.* 2015; **128**: 298–305.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

18. Baum J, Papenfuss AT, Mair GR, *et al.*: **Molecular genetics and comparative genomics reveal RNAi is not functional in malaria parasites.** *Nucleic Acids Res.* 2009; **37**(11): 3788–3798.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

19. Hentzschel F, Mitesser V, Fraschka SA, *et al.*: **Gene knockdown in malaria parasites via non-canonical RNAi.** *Nucleic Acids Res.* 2020; **48**(1): e2.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

20. Brancucci NMB, Goldowitz I, Buchholz K, *et al.*: **An assay to probe** *Plasmodium falciparum* **growth, transmission stage formation and early gametocyte development.** *Nat Protoc.* 2015; **10**(8): 1131–1142.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

21. Tonkin CJ, van Dooren GG, Spurck TP, *et al.*: **Localization of organellar proteins in** *Plasmodium falciparum* **using a novel set of transfection vectors and a new immunofluorescence fixation method.** *Mol Biochem Parasitol.* 2004; **137**(1): 13–21.
    PubMed Abstract | **Publisher Full Text**

22. Schindelin J, Arganda-Carreras I, Frise E, *et al.*: **Fiji: an open-source platform for biological-image analysis.** *Nat Methods.* 2012; **9**(7): 676–682.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

23. Schneider CA, Rasband WS, Eliceiri KW: **NIH Image to ImageJ: 25 years of image analysis.** *Nat Methods.* 2012; **9**(7): 671–675.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

24. Tsuji M, Mattei D, Nussenzweig RS, *et al.*: **Demonstration of heat-shock protein 70 in the sporozoite stage of malaria parasites.** *Parasitol Res.* 1994; **80**(1): 16–21.
    PubMed Abstract | **Publisher Full Text**

25. Mantel PY, Hoang AN, Goldowitz I, *et al.*: **Malaria-infected erythrocyte-derived microvesicles mediate cellular communication within the parasite population and with the host immune system.** *Cell Host Microbe.* 2013; **13**(5): 521–534.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

26. Hentzschel F, Marti M, Obrová K: **Extended Figures 1 and 2.** *figshare.* Figure. 2020.
    http://www.doi.org/10.6084/m9.figshare.12229028.v1

27. Hentzschel F, Marti M, Obrová K: **No evidence for Ago2 translocation from the host erythrocyte to the** *Plasmodium* **parasite.** *figshare.* Collection. 2020.
    http://www.doi.org/10.6084/m9.figshare.c.4960649.v1

28. Masciarelli S, Quaranta R, Iosue I, *et al.*: **A small-molecule targeting the microRNA binding domain of argonaute 2 improves the retinoic acid differentiation response of the acute promyelocytic leukemia cell line NB4.** *ACS Chem Biol.* 2014; **9**(8): 1674–1679.
    PubMed Abstract | **Publisher Full Text**

29. Elahi R, Dapper C, Klemba M: **Internalization of Erythrocyte Acylpeptide Hydrolase Is Required for Asexual Replication of** *Plasmodium falciparum*. *mSphere.* 2019; **4**(3): e00077-19.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

30. Desai SA, Krogstad DJ, McCleskey EW: **A nutrient-permeable channel on the intraerythrocytic malaria parasite.** *Nature.* 1993; **362**(6421): 643–646.
    PubMed Abstract | **Publisher Full Text**

31. Mesén-Ramírez P, Bergmann B, Tran TT, *et al.*: **EXP1 is critical for nutrient uptake across the parasitophorous vacuole membrane of malaria parasites.** *PLoS Biol.* 2019; **17**(9): e3000473.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

32. Birnbaum J, Scharf S, Schmidt S, *et al.*: **A Kelch13-defined endocytosis pathway mediates artemisinin resistance in malaria parasites.** *Science.* 2020; **367**(6473): 51–59.
    PubMed Abstract | **Publisher Full Text**

33. Koncarevic S, Rohrbach P, Deponte M, *et al.*: **The malarial parasite** *Plasmodium falciparum* **imports the human protein peroxiredoxin 2 for peroxide detoxification.** *Proc Natl Acad Sci U S A.* 2009; **106**(32): 13323–8.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

34. Leung AKL, Sharp PA: **Quantifying Argonaute proteins in and out of GW/P-bodies: implications in microRNA activities.** *Adv Exp Med Biol.* In: *Ten Years of Progress in GW/P Body Research.* (Springer, New York, NY). 2013; **768**: 165–182.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

35. Leung AKL: **The Whereabouts of microRNA Actions: Cytoplasm and Beyond.** *Trends Cell Biol.* 2015; **25**(10): 601–610.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

36. Jee D, Lai EC: **Alteration of miRNA activity via context-specific modifications of Argonaute proteins.** *Trends Cell Biol.* 2014; **24**(9): 546–553.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

37. Zeng Y, Sankala H, Zhang X, *et al.*: **Phosphorylation of Argonaute 2 at serine-387 facilitates its localization to processing bodies.** *Biochem J.* 2008; **413**(3): 429–436.
    PubMed Abstract | **Publisher Full Text**

38. Van Eijl RAP, Van Den Brand T, Nguyen LN, *et al.*: **Reactivity of human AGO2 monoclonal antibody 11A9 with the SWI/SNF complex: A case study for rigorously defining antibody selectivity.** *Sci Rep.* 2017; **7**(1): 7278.
    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 13 January 2021

https://doi.org/10.21956/wellcomeopenres.18055.r41546

✔ **Tim W. Gilberger**
   [1] Department of Biology, University of Hamburg, Hamburg, Germany
   [2] Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany

The authors addressed all my comments and I approve the revised version!

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plasmodium falciparum cell biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 04 December 2020

https://doi.org/10.21956/wellcomeopenres.18055.r41547

✔ **Julian Rayner** iD
   Cambridge Institute for Medical Research, University of Cambridge, Cambridge, UK

The repetition of fractionation with the same strain as used for the IFA rules out any (always likely limited) chance of strain-specific differences, and the additional details further clarify the finding. No further comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Malaria parasite cell biology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 16 July 2020

https://doi.org/10.21956/wellcomeopenres.17389.r39405

? **Tim W. Gilberger**
[1] Department of Biology, University of Hamburg, Hamburg, Germany
[2] Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany

The manuscript by Hentzschel *et al.* revisited the localization and putative function of human Ago2 in Plasmodium-infected erythrocytes. Recent studies, including a paper published in 2016 by the authors, suggested that hAgo2 can translocate into the parasite and affect its development. This prior work was partially based on IFA using a commercial monoclonal.
Now the authors re-analyzed this localization by a comparative approach using several different monoclonal antibodies in *P. falciparum* and *P. berghei* infected erythrocytes and probed the function of hAgo2 in parasite maturation using the Ago2 inhibitor BCI-137.

They show ambiguous localization patterns in IFAs within the parasite that point towards cross-reactivity of the anti-Ago2 antibodies and does not support uptake of hAgo2 into the parasite. This finding was further supported by cell fractionation of parasite-infected erythrocytes that showed Ago2 only in the cytoplasm of the erythrocytes and not in the parasite. Additionally, the BCl-137-based Ago2 inhibition over a wide concentration range did not result in altered parasite development.

In my view, this work is an important contribution to the field because it sets the record straight using complementary techniques. It also highlights the challenges of using IFAs and the need for a very thorough validation of the antibodies used for protein localization.

I have only a few additional comments on this well performed study:

○ In their original study, the authors used 3D7 and show the staining of ring stage parasites by the monoclonal. In Fig. 2 C they provide their comparative localization study with Pf2004 parasites. Although I very much doubt that there are any strain specific differences, for the sake of a direct comparison, 3D7 based localization would be beneficial. Side note: I could not find a reference for the Pf2004 parasite strain.

○ Ago2 inhibition (Fig. 4) – Granting that the authors are using BCl-137 in wide molar range, without a positive control for BCL-137-based inhibition of Ago2, this data point remains debatable.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plasmodium falciparum cell biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 01 June 2020

https://doi.org/10.21956/wellcomeopenres.17389.r38705

? **Julian Rayner** (iD)
Cambridge Institute for Medical Research, University of Cambridge, Cambridge, UK

This study expands on previous work by both the lead authors and others that identified a potential role for human Argonaute2 (Ago2), a protein involved in RNAi, in *Plasmodium falciparum* intraerythrocytic development. Two previous manuscripts established the presence of Ago2 in extracellular vesicles (EVs) released from *P. falciparum* infected erythrocytes, where it could

complex with microRNA and impact endothelial cell function. Both studies also reported the presence of Ago2 in *P. falciparum* parasites, rather than solely in the erythrocyte cytosol and released EVs, and some of this previous data implied that Ago2 might play a direct role in regulating *P. falciparum* development. In this study the authors compared multiple different commercial anti-Ago2 antibodies in both immunofluorescence and immunoblot experiments and the results suggested that the previous evidence for Ago2 localisation in *P. falciparum* parasites was due to antibody cross-reaction and incomplete fractionation. An Ago2 inhibitor had no impact on parasite development, and the authors therefore argue that there is no conclusive evidence for Ago2 activity in *P. falciparum* parasites.

While this kind of study is perhaps unglamorous, it is an important addition to the scientific literature as it clarifies previous data, including data previously published by the authors themselves, and might prevent others pursuing hypotheses that are unlikely to be fruitful. It's indexing is therefore strongly supported. The experiments are generally clear and compelling, and the inclusion of full-length blots as extended figures are an example of publishing best practise that should be commended. While the overall case is clear, some minor details and controls would perhaps complete the story and completely show that the earlier indications of Ago2 functioning within *P. falciparum* parasites were misleading.

1. The authors show that three different anti-Ago2 antibodies result in different, non-overlapping, staining patterns in *P. falciparum* infected erythrocytes, compelling evidence that they are recognising additional, non-Ago2, proteins at the dilutions used for immunofluorescence. The comparability of these data with the previous publications is clearly key. While one of the three antibodies, 2E12-1C9, is the same monoclonal used in both previous studies, each study used 2E12-1C9 from different suppliers. To rule out any possible confounding explanation for the different results (such as antibody mix up from certain suppliers), is it not possible to source exactly the same antibodies used in previous studies and compare them?

2. Both previous studies used the *P. falciparum* 3D7 strain, but the key figures in this work used a different strain, Pf2004. While it would seem unlikely that Ago2 would play a strain-specific role in parasite development, for completeness sake it would be useful to rule it out, for example by repeating the fractionation data in Figure 3 (which comprehensively argues against the presence of Ago2 in *P. falciparum* parasites) with 3D7.

3. As noted above, Figure 3 is the most convincing data to rule out Ago2 in the parasite, but the methodology used differs subtly from the author's previously published fractionation (ref 5), which used sequential tetanolysin then saponin fractionation. That approach indicated Ago2 was found in the parasite at the 24 hour time point only, and was found in the parasitophorous vacuole throughout parasite development. The authors argue that their previous data indicating Ago2 in the parasite was the result of incomplete fractionation, but what about the presence of Ago2 in the parasitophorous vacuole - is this still possible?

4. Given the clear evidence of cross-reactivity of anti-Ago2 antibodies with a *P. falciparum* protein(s), and the presence of a few faint parasite bands recognised by anti-Ago2 antibodies in the extended Figures, is there any indication what these cross-reacting proteins might be? If you BLAST a Pf database with just the segments of Ago2 used to raise antibodies, does anything come up at a convincing level of homology?

5. The BCI-137 inhibition data is clearly negative, but lacks a positive control to show that the concentrations used of this inhibitor from this supplier are active, for example by showing enzymatic inhibition, or inhibition of some other non-*Plasmodium* cell.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Malaria parasite cell biology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.