SUPPLEMENTAL INFORMATION

***Plasmodium falciparum* sexual parasites develop in human erythroblasts and affect erythropoiesis**

Gaëlle Neveu, Cyrielle Richard, Florian Dupuy, Prativa Behera, Fiona Volpe, Pradeep Annamalai Subramani, Benjamin Marcel-Zerrougui, Patrice Vallin, Muriel Andrieu, Aruna Mukti Minz, Nabih Azar, Rafael M. Martins, Audrey Lorthiois, Florence Gazeau, José-Juan Lopez-Rubio, Dominique Mazier, Amanda K. A. Silva, Sanghamitra Satpathi, Samuel C. Wassmer, Frédérique Verdier, Catherine Lavazec.

SUPPLEMENTAL METHODS

Parasites

The *P. falciparum* NF54 clone B10[1] was used to generate the transgenic NF54-pfs47-Hsp70-GFP line (called Hsp70-GFP) and the NF54-pfs47-Pfs16-GFP line (called Pfs16-GFP) described below. The VarO gametocytes non-producing line was described elsewhere[2]. Parasites were cultivated *in vitro* as described[3] using RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated human serum, hypoxanthine (10mM), gentamicin (20µg/ml) and human erythrocytes at 5% hematocrit. Infected erythrocytes were cultivated at 37°C, 5% CO2, 5% O2. To obtain synchronous asexual stages, trophozoites were isolated by magnetic isolation using a MACS depletion column (Miltenyi Biotec) in conjunction with a magnetic separator, and placed back into culture. 3-4 hours after the invasion of merozoites, ring-stage parasites were selected by magnetic depletion of schizonts. Synchronous production of gametocytes in erythrocytes was achieved by treating synchronized asexual cultures at the ring stage (15-20 % parasitemia) with 50 mM N-acetylglucosamine for 5 days. To obtain conditioned medium, medium was collected from a NF54 parasite culture between 5 to 10 % parasitemia cultivated overnight in complete parasite medium. Medium specifically conditioned by asexual parasites was collected from a culture of the VarO line, which has lost the ability to produce gametocytes. Medium

specifically conditioned by immature gametocyte-infected erythrocytes was collected from a synchronized gametocyte culture 5 to 6 days after adding N-acetylglucosamine.

Generation of the Hsp70-GFP and the Pfs16-GFP transgenic lines

To generate the NF54-pfs47-hsp70-GFP line (called Hsp70-GFP line in the text) expressing GFP under the control of the constitutive promoter *hsp70* and the NF54-pfs47-pfs16-GFP line (called Pfs16-GFP line in the text) expressing GFP under the control of the gametocyte-specific promoter *pfs16*, cultures of the NF54 clone B10 were co-transfected with 70 µg of plasmid pDC2-Cas9-hDHFRyFCU and 70 µg of plasmid pBLD588-hsp70-GFP or 70 µg of plasmid pBLD588-pfs16-GFP and selected with 2.5 nM WR99210 as previously described[4]. The plasmid pDC2-Cas9-hDHFRyFCU[5] encodes a single guide RNA that recognizes a sequence located in the *pfs47* locus. Both the plasmid pBLD588-Hsp70-GFP and the plasmid pBLD588-pfs16-GFP contain two homology regions, HR1 and HR2, corresponding to the sequences in the *pfs47* gene and upstream of the *pfs47* gene, respectively. For the plasmid pBLD588-Hsp70-GFP, HR1 and HR2 flank the *hsp70* 5'flanking region followed by the *gfp* coding sequence and the PbDT 3' regulatory region. For the plasmid pBLD588-pfs16-GFP, HR1 and HR2 flank the *pfs16* 5'flanking region[6] followed by the *gfp* coding sequence and the PbDT 3' regulatory region. Details about the generation of the plasmids and PCR validation of the NF54-pfs47-Hsp70-GFP and of the NF54-pfs47-pfs16-GFP are provided in Figure S2 and Figure S3, respectively. Following transfection and drug selection, clones were obtained by limiting dilution.

Human primary erythroblasts

The human primary erythroblasts culture method is fully described in Gautier et al.[7]. Briefly, CD34+ progenitor cells were obtained from human donors who gave informed consent in accordance with the Declaration of Helsinki Principles. The study has been approved by the INSERM Institutional Review Board IRB 00003888. CD34+ cells were collected from bone marrow aspirate or G-CSF mobilized peripheral blood after cytapheresis and purified by an immunomagnetic procedure (MACS CD34

isolation Kit; Miltenyi Biotec). CD34+ cells were cultured at 37°C, 5% CO2, in Iscove DMEM (IMDM) containing 15 % BIT 9500 (StemCell Technologies), Stem Cell Factor (100 ng/mL) (SCF, Milteny Biotec), IL6 (10 ng/ml) and IL3 (10 ng/ml) (Milteny Biotec). After 7 days of culture, a purified population of human erythroid progenitors was obtained by positive selection on CD36 immunomagnetic beads (CD36 FA6.152, Beckman Coulter). CD36+ cells were cultured with Erythropoietin (EPO) (2 U/mL), SCF (100 ng/mL), IL3 (10 ng/mL) and dexamethasone (2.10^{-7}M) (Sigma) during 5 days for progenitor amplification. To synchronously induce terminal differentiation, cells were washed with Phosphate Buffer Saline (PBS) and cultivated for 24 hours with RU-486 (3 µM) (Sigma) to block dexamethasone effects, SCF (40 ng/ml), EPO (4 U/ml) and heat-inactivated human serum (3%). Then, cells were daily diluted to 0.8 million cells/ml with culture medium containing 3% heat-inactivated human serum and EPO only.

Characterization of erythroid differentiation

Erythroid differentiation was analyzed by May-Grünwald-Giemsa (MGG) staining of cytospun samples and by flow cytometry using the following antibodies: PE-conjugated anti-CD34 (1/50), FITC-conjugated anti-CD36 (1/100), FITC-conjugated anti-CD71 (1/50), PC7-conjugated anti-GPA (1/50), APC-conjugated anti-CD49d (α4-integrin) (1/10) (all from Beckman Coulter) and PE-conjugated anti-BRIC6 (anti-Band 3) (1/200) (International Blood Group Reference Laboratory NHS Blood and Transplant). Reticulocytes production was monitored using the DNA-labelling Draq5 probe (1/5000) (Thermofisher).

Elaboration of the conditioned medium

To generate parasite-conditioned medium from erythrocytes culture (iECM) or erythroblasts culture (iEbCM), cultures between 5 to 10% parasitemia (or 0.5, 2 and 20% if specified) were cultivated during 24 hours at 5% hematocrit with erythroblasts culture medium adapted to parasites: IMDM containing 15% BIT 9500, 1% glutamine, 1% penicillin-streptomycin, EPO (4U/ml), hypoxanthine (10mM) and heat-inactivated human serum (10%). After incubation, the conditioned medium was centrifuged 15 minutes

at 600, 1,600 and 3,600*xg* to eliminate cells from the supernatant. Conditioned medium was filtered at 0.8µM and stored at 4°C before analysis.

Purification of extracellular vesicles (EVs)

Protocol for EVs purification was adapted from[8]. Briefly, a parasite culture between 5 to 10 % parasitemia was cultivated overnight in complete parasite medium with human serum depleted in EVs (HS - EVs). To prepare HS - EVs, human serum was ultracentrifuged 2 hours at 100,000*xg* and the supernatant was filtered at 0.1 µM. The supernatant of the parasite culture was centrifuged 15 minutes at 600, 1,600 and 3,600*xg* before a 1 hour-ultracentrifugation step at 100,000*xg*. After ultracentrifugation, the supernatant was filtered at 0.1 µM, leading to iECM depleted in EVs (iECM - EV). The pellet was washed with 1X PBS and ultracentrifuged 1 hour at 100,000*xg*. EVs in the pellet were carefully resuspended in 1X PBS and layered on the top 60% sucrose solution and centrifuged for 1 hour at 100,000*xg* with low acceleration and low brake at 4°C. EVs were collected at the interphase on top of the sucrose and washed in 1X PBS. EVs amount was determined by protein content using a Pierce BCA protein assay kit (Thermofisher).

Purified EVs and conditioned medium were labeled with PC7-conjugated anti-GPA (1/10) (Beckman Coulter) or with the isotypes-PC7 conjugated monoclonal antibody (1/10) (Beckman Coulter). EVs were analyzed with MoFlo Astrios (Beckman Coulter, Brea, CA) equipped with a PhotoMulTiplicator (PMT)-Forward SCatter (FSC) at CYBIO core facility (Cochin Institute, Paris). Flow cytometer performance was assessed before cell analyzing experiments. Polystyrene beads called Megamix (BioCytex) of known dimensions (100, 300, 500 and 900nm in diameter) were used to standardize PMT-FSC parameters and to define the total EV gate. Side SCatter (SSC) signals were acquired on both 488 and 561nm lasers. The same time speed of acquisition was used in all cases to compare samples. Total EVs were determined and counted on the basis of size (between 100 and 600nm), and the number and percentage of GPA positive particles. Flow cytometry data were analyzed with FlowJo software (vX, Ashland, OR).

Nanoparticle tracking analysis

Two samples of purified EVs (samples A and B) were analyzed by Nanoparticle analysis (NTA). NTA measurements were carried out using a Nanosight NS300 (Malvern, UK) equipped with a 405 nm laser, a sCMOS camera and a syringe pump. Samples were diluted in PBS. A dilution factor of 333 and 400 was used for samples A and B, respectively, in order to obtain 20–100 particles/frame. PBS was also analyzed as a control. The following settings were used for the experiments: camera level of 16 and detection threshold of 5. For each measurement, five videos were captured at 25°C with a minimal number of 4865 completed tracks per video and a total of 1498 frames analyzed per video. The analysis of the videos was carried out by the NanoSight Software NTA version 3.3, particle diameter being calculated from Stokes-Einstein equation. Sample featured characteristic EV size distribution mainly ranging from 70 to 200 nm, with a main peak near 130 nm. For sample A, mean size and mode were 132.6 ± 2.4 nm and 89.6 ±3.6 nm, respectively, with a size distribution standard deviation of 64.5 +/- 3.9 nm. For this sample, 90% of the particles were smaller than 202.1 ± 8.6 nm, while 10% of the particles were smaller than 84.0 ± 1.9 nm. Sample A concentration was $2.8 \times 10^8 \pm 2.5 \times 10^7$ particles/ml following a 333-fold dilution in PBS. For sample B, mean size and mode were 126.9 ± 3.4 and 107.7 ± 5.2 nm, respectively, with a size distribution standard deviation of 39.5 ± 1.6 nm. For this sample, 90% of the particles were smaller than 169.4 ± 6.0 nm, while 10% of the particles were smaller than 90.2 ± 2.6 nm. Sample B concentration was $2.8 \times 10^8 \pm 1.8 \times 10^7$ particles/ml following a 400-fold dilution in PBS. For PBS control, the measured concentration was $3.8 \times 10^6 \pm 1.0 \times 10^6$ particles/ml.

Electron microscopy

Erythroblasts infected at 6 dpi were washed twice with 0.1 M phosphate buffer, pH 7.2 and then fixed with 2% paraformaldehyde (PFA), 2% glutaraldehyde, 1 mM calcium chloride in 0.1 M phosphate buffer, pH 7.2 for 10 minutes at room temperature. Afterwards, the fixed cells were embedded in 4% low melting agarose and either stored at 4°C until further use or processed immediately by washing 5 times

with 0.2 M cacodylate buffer, pH 7.4 and fixed with 2% Osmium tetroxide for 1 hour and washed 5 times with water, followed by fixation with 5% uranyl acetate for 1 hour and washed 5 times with water. Next, cells were dehydrated in 50, 75, 90, 95% and absolute ethanol, saturated with a mixture of epoxy resin and ethanol (50:50) overnight, followed by embedding in epoxy resin at 60°C for 48 hours. Resin blocks were then cut with an ultra-microtome to generate 500 nm semi-thin sections that were stained with toluidine blue to assess the quality of samples and identify the area of interest. The selected region of interest was further cut into 70 nm ultra-thin sections and collected on copper grids, treated with lead citrate for 5 minutes and taken for electron microscopy for imaging on the microscope Hitachi Transmission Electron Microscopy HT7700.

EVs were incubated on Formvar® coated grids for 2 minutes and then with uranyl acetate 1% for 2 minutes. EVs were directly observed with a JEOL 1011 transmission electron microscope. Acquisitions were performed with a GATAN Orius 1000 camera.

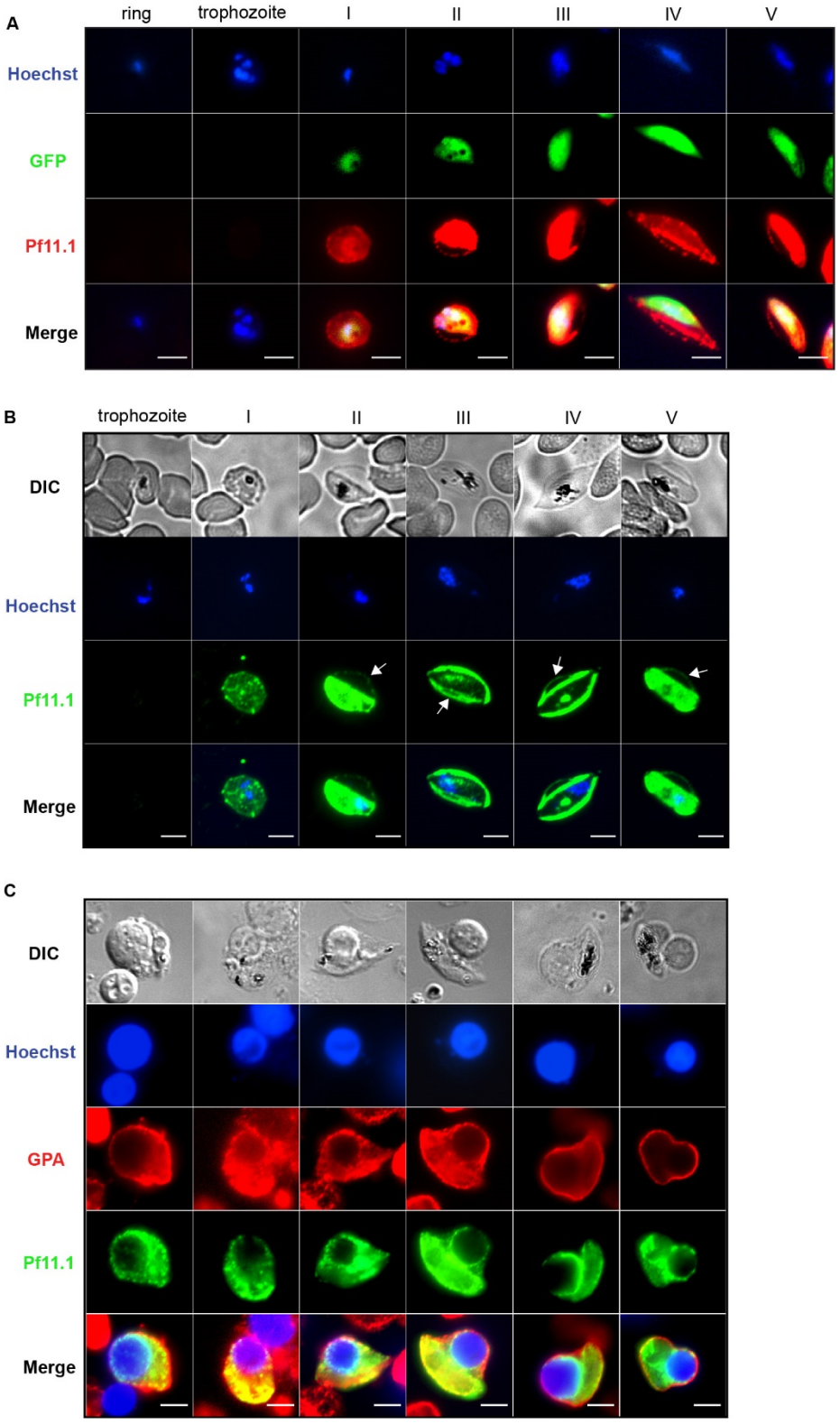
Fluorescent microscopy

Infected erythroblasts were fixed during 10 minutes with a solution of 1X PBS/4% PFA. Then, cells were stained with Hoechst 33342 (Thermofisher) (1/20000) and PKH26 (Sigma) according to manufacturer's instructions, PKH26 is a red fluorescent probe that label lipid membranes. Cells were permeabilized if needed in a solution of 0.1% Triton in 1X PBS 10 minutes at room temperature. For immunofluorescence, cells or bone marrow smears were pre-incubated 2 hours in 1X PBS/2% Bovine Serum Albumin (BSA) and incubated overnight with a mouse monoclonal antibody directed against Glycophorin A (Santa Cruz) (1/500) and with rabbit antibody raised again the Pf11-1 protein (1/2000)[9]. Then, cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit (1/2000) and Alexa Fluor 594-conjugated goat anti-mouse antibody (1/2000) (Thermofisher) and Hoechst 33342 (1/20000) for 1 hour at room temperature. All samples were observed at X100 magnification using a Leica DMi8 microscope.

Quantification and statistical analysis

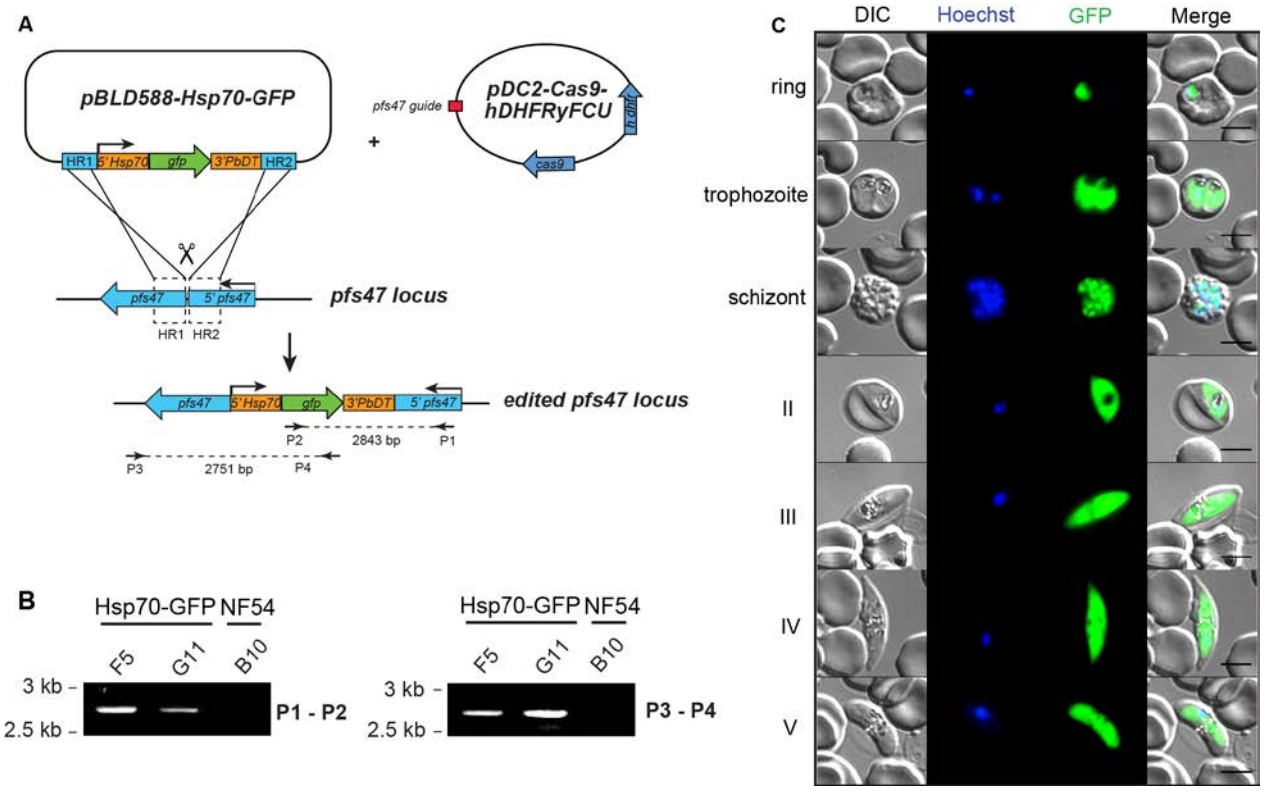
Statistical analyses were performed with GraphPad Prism 7. To compare two conditions, statistical significance was determined by Mann-Whitney test or by paired *t* test for two populations within the same culture. To compare more than 2 conditions, statistical significance was determined by one-way ANOVA test. Sidak corrections for multiple comparisons were applied when different pairs of conditions were compared together. Dunnett corrections for multiple comparisons were applied when the means of each condition were compared to the mean of the control condition.

SUPPLEMENTAL FIGURES AND LEGENDS



Supp. Figure S1. Related to Figure 1.

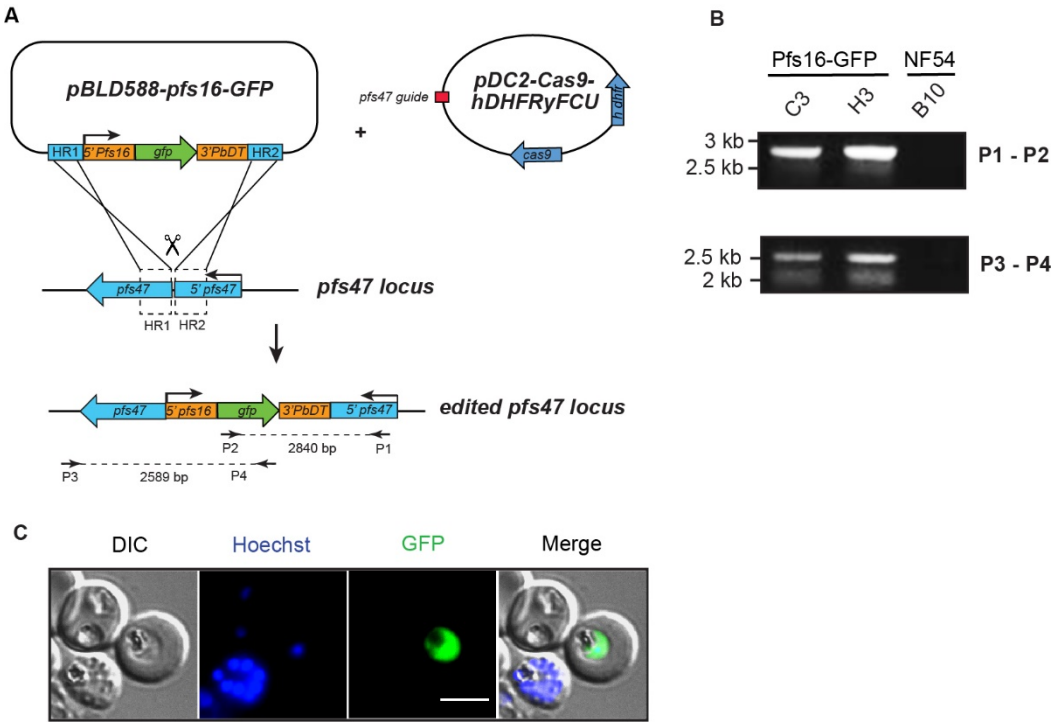
(A) IFA of the Pfs16-GFP line showing the expression of the gametocyte specific protein Pf11-1 (red) during gametocytogenesis. Pf11-1 is not expressed in asexual stages. (B) IFA of NF54 gametocytes from stage I to stage V showing the export of Pf11-1 (green) to the erythrocyte membrane (arrows). (C) IFA of infected erythroblasts at 6 dpi stained with anti-Pf11-1 antibodies (green) and with anti-glycophorin A (GPA) labelling the erythroblast membrane (red). DNA is stained with Hoechst 33342 (blue). Bars represent 5 μm.



Supp. Figure S2. Related to Figure 1.

Generation of parasites expressing GFP under the control of the *hsp70* promoter using CRISPR-Cas9 technology. (A) Schematic (not to scale) of the GFP insertion into the *pfs47* locus. The scissors indicate the Cas9 cleavage site. Genomic DNA from NF54 parasites was used for the amplification of 5'*hsp70* with P5 and P6 primers. The *gfp* sequence was amplified from EGFP-Vimentin-7 plasmid (Addgene) with P2 and P4 primers. Generation of pBLD588-hsp70-GFP was performed by a double digestion of the pBLD588 plasmid with SpeI and XhoI restriction enzymes. Both 5'*hsp70* and *gfp* fragments were then cloned in frame using the In-Fusion system (Ozyme). (B) PCR analysis of genomic DNA from F5 and G11 clones obtained by limiting dilution of the NF54-pfs47-hsp70-GFP line. The NF54 clone B10 was used as control. Arrows indicate the position of primers used to confirm plasmid integration into the *pfs47* locus. P1: CCTAACACATTATGTGTATAACATTTTATGC, P2: AAGCTTATGGTGAGCAAGGGCGAGGAG, P3: GCGATATGTAATTCCATTACTGC, P4: CGTTATGTTACTCGAGTTACTTGTACAGCTCGTCCATGCC, P5: TTTATAGTACACTAGTACAATATGAATTTATAGAGCAAATTTATATAG, P6:

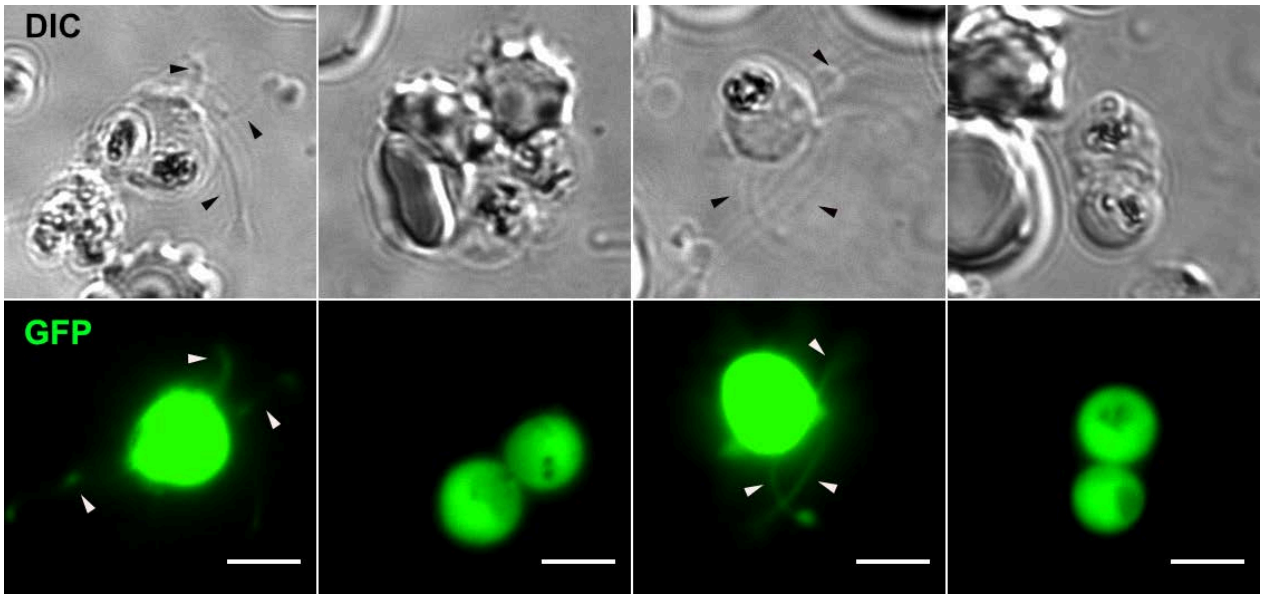
GCTCACCATAAGCTTGTTGAAGAAAGTATAAATAGAAAAATGG. (C) Fluorescence microscopy analysis of the clone F5 showing GFP in all asexual and sexual stages. DNA is stained with Hoechst 33342 (blue). Scale bars: 5 µm.



Supp. Figure S3. Related to Figure 1.

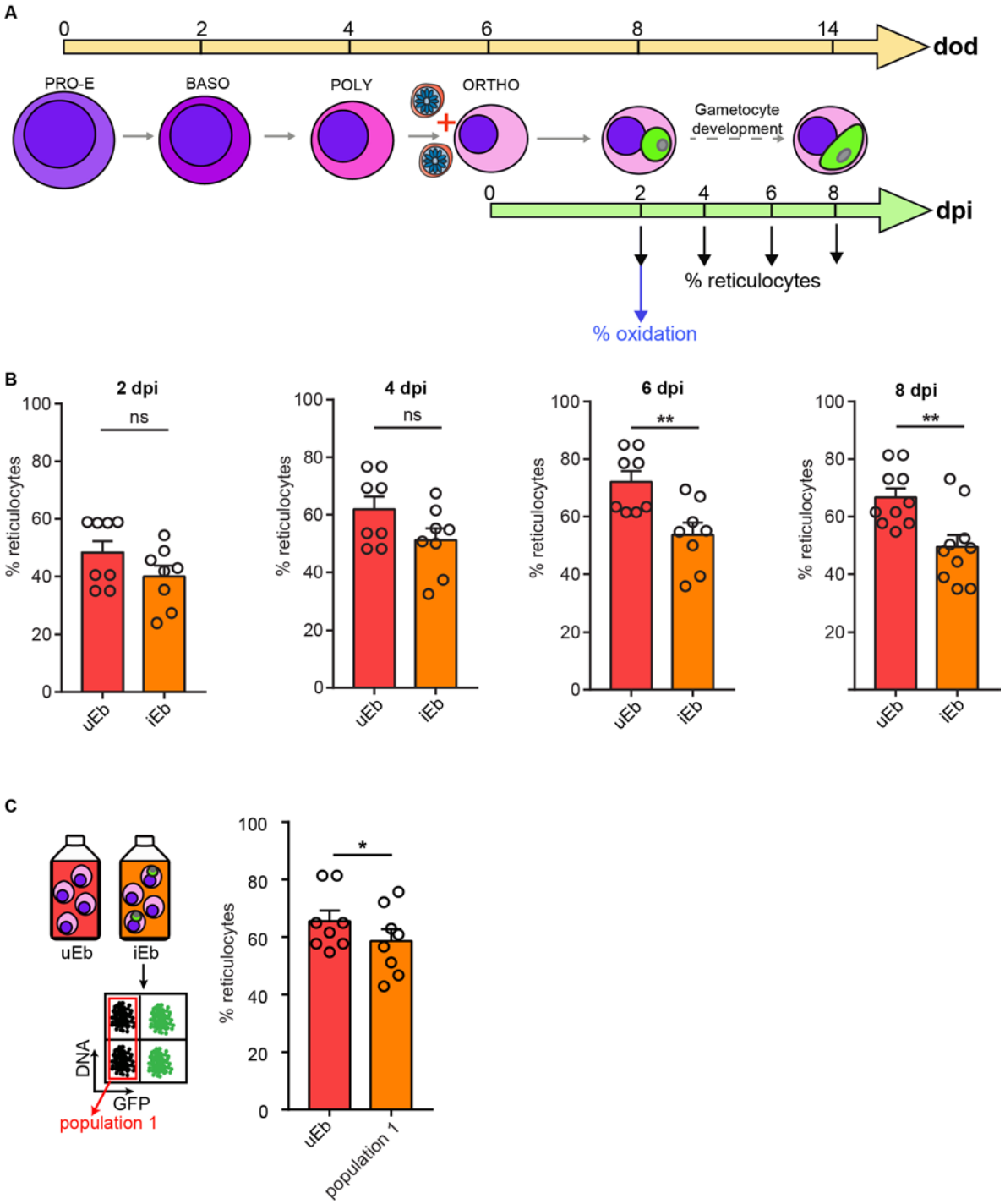
Generation of parasites expressing GFP under the control of the gametocyte-specific promoter of *pfs16* using CRISPR-Cas9 technology. (A) Schematic (not to scale) of the GFP insertion into the *pfs47* locus. The scissors indicate the Cas9 cleavage site. Genomic DNA from NF54 parasites was used for the amplification of 5' *pfs16* with P7 and P8 primers. The *gfp* sequence was amplified from EGFP-Vimentin-7 plasmid (Addgene) with P2 and P4 primers. Generation of pBLD588-pfs16-GFP was performed by a double digestion of the pBLD588 plasmid with SpeI and XhoI restriction enzymes. Both 5'*pfs16* and *gfp* fragments were then cloned in frame using the In-Fusion system (Ozyme). (B) PCR analysis of genomic DNA from C3 and H3 clones obtained by limiting dilution of the NF54-pfs47-pfs16-GFP line. The NF54 clone B10 was used as control. Arrows indicate the position of primers used to confirm plasmid

integration into the *pfs47* locus. P7: TTTATAGTACACTAGTACAATATGAATTTATAGAGCAAATTTATATAG; P8: GCTCACCATAAGCTTGTTGAAGAAAGTATAAATAGAAAAATGG. (C) Fluorescence microscopy analysis of the clone C3 showing GFP in stage I gametocytes. DNA is stained with Hoechst 33342 (blue). Scale bar: 5 µm.



Supp. Figure S4. Related to Figure 2.

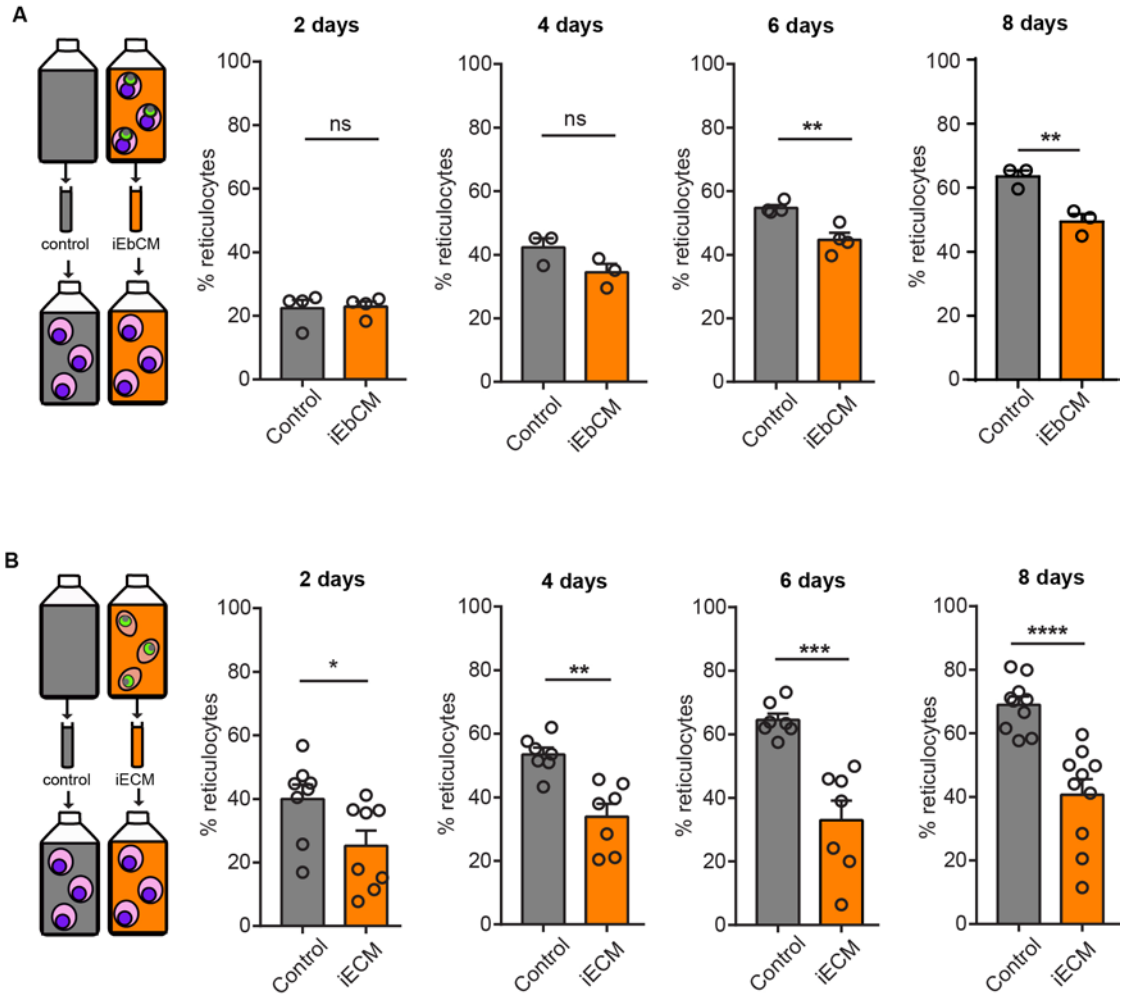
Fluorescence microscopy of male and female activated gametes from a culture of infected erythroblasts with the Pfs16-GFP line. Arrows indicate flagella. Bars represent 5 µm.



Supp. Figure S5. Related to Figure 5.

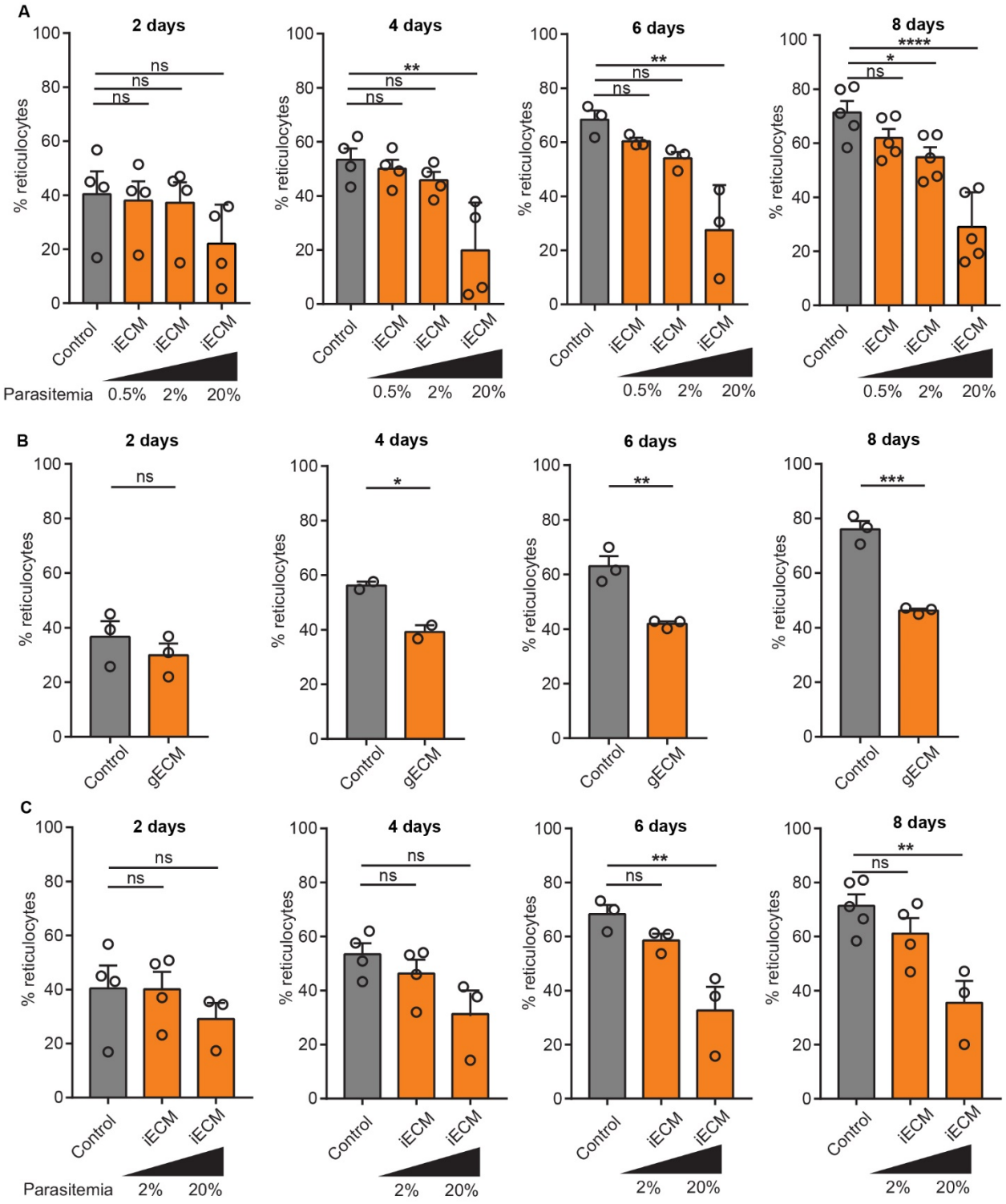
(**A**) Diagram illustrating the erythroblast infection protocol and subsequent measurement of oxidation and reticulocyte rate. After 6 days of differentiation (dod) from pro-erythroblast (PRO-E) to orthochromatic (ORTHO) stages, synchronized schizonts were added to erythroblasts. The percentage of

reticulocytes is monitored in the culture by flow cytometry at 2, 4, 6 and 8 days post infection (dpi). The percentage of oxidized erythroblasts is monitored in the culture by flow cytometry at 2 dpi. (B) Percentage of reticulocytes in infected (iEb) or not infected (uEb) erythroblast culture, is evaluated by flow cytometry 2 days, 4 days, 6 days and 8 days after the infection. (C) Percentage of reticulocytes in an uninfected erythroblast culture (uEb) or in the population of non-infected cells within a Pfs16-GFP-infected culture (population 1) at 8 dpi. Circles indicate the number of independent experiments and error bars show the SEM. * $p < 0.05$, ** $p < 0.01$, ns: non-significant difference.



Supp. Figure S6. Related to Figure 5.

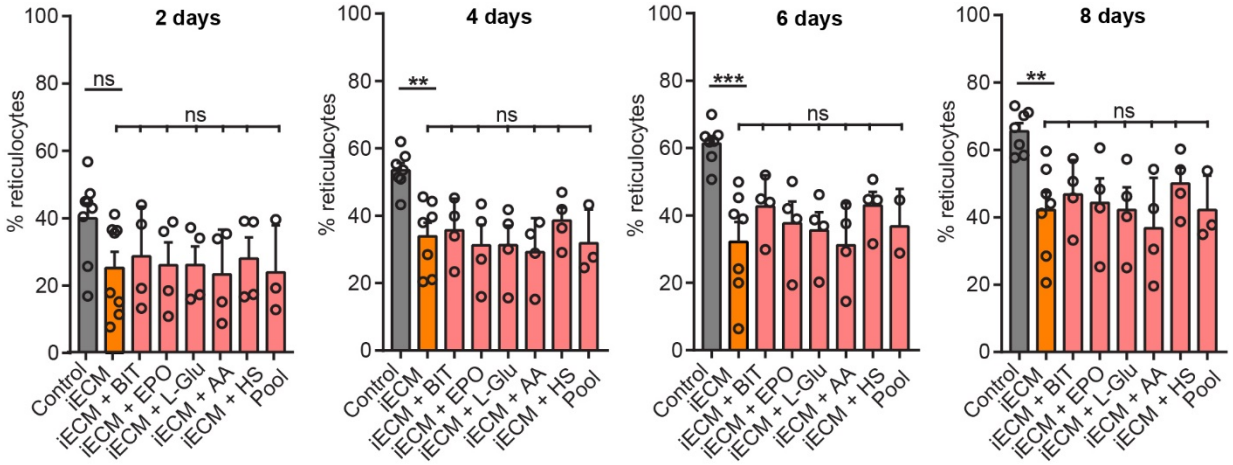
(A and B) Percentage of reticulocytes in erythroblast culture at 2 days, 4 days, 6 days and 8 days after addition of conditioned medium obtained with a culture of infected erythroblasts (iEbCM, A), infected erythrocytes (iECM, B), or control medium (Control). Circles indicate the number of independent experiments and error bars show the SEM. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns: non-significant difference.



Supp. Figure S7. Related to Figure 5.

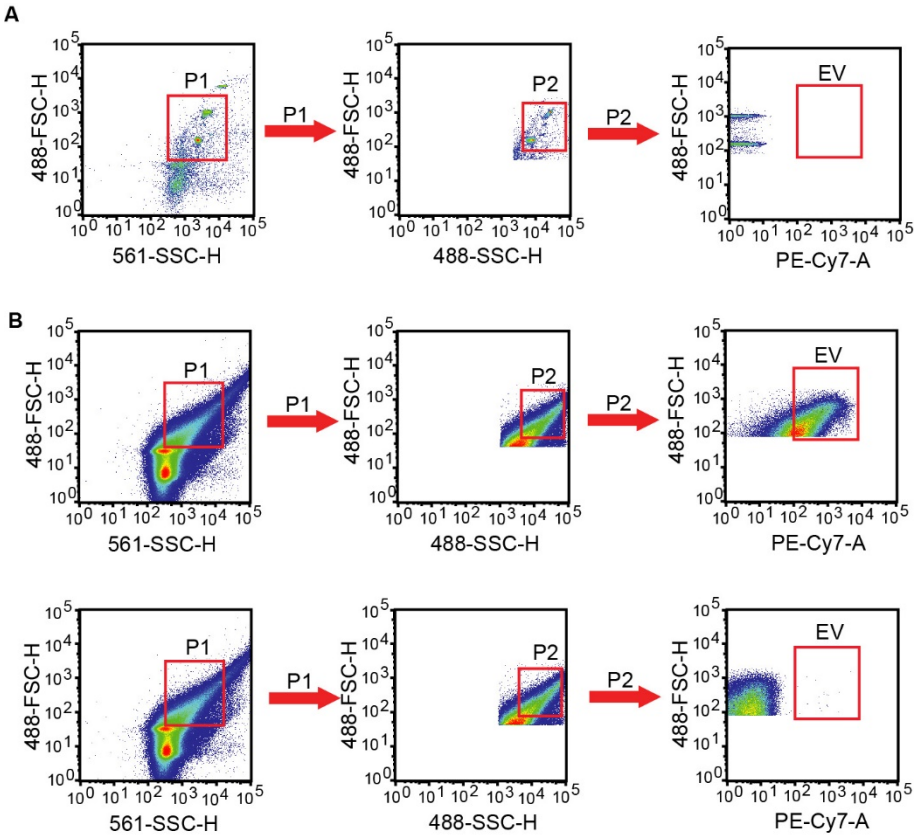
(A) Percentage of reticulocytes in erythroblast culture at 2 days, 4 days, 6 days and 8 days after addition of iECM obtained with parasite cultures at 0.5%, 2% and 20% parasitemia, or control medium. (B)

Percentage of reticulocytes in erythroblasts culture at 2 days, 4 days, 6 days and 8 days after addition of medium conditioned by a culture of erythrocytes infected by gametocytes (gECM) or control medium (control). (C) Percentage of reticulocytes in erythroblasts culture at 2 days, 4 days, 6 days and 8 days after addition of iECM obtained with parasite cultures at 2 % and 20 % parasitemia of the gametocytes non-producing line VarO line, or control medium (control). Circles indicate the number of independent experiments and error bars show the SEM. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns: non-significant difference.



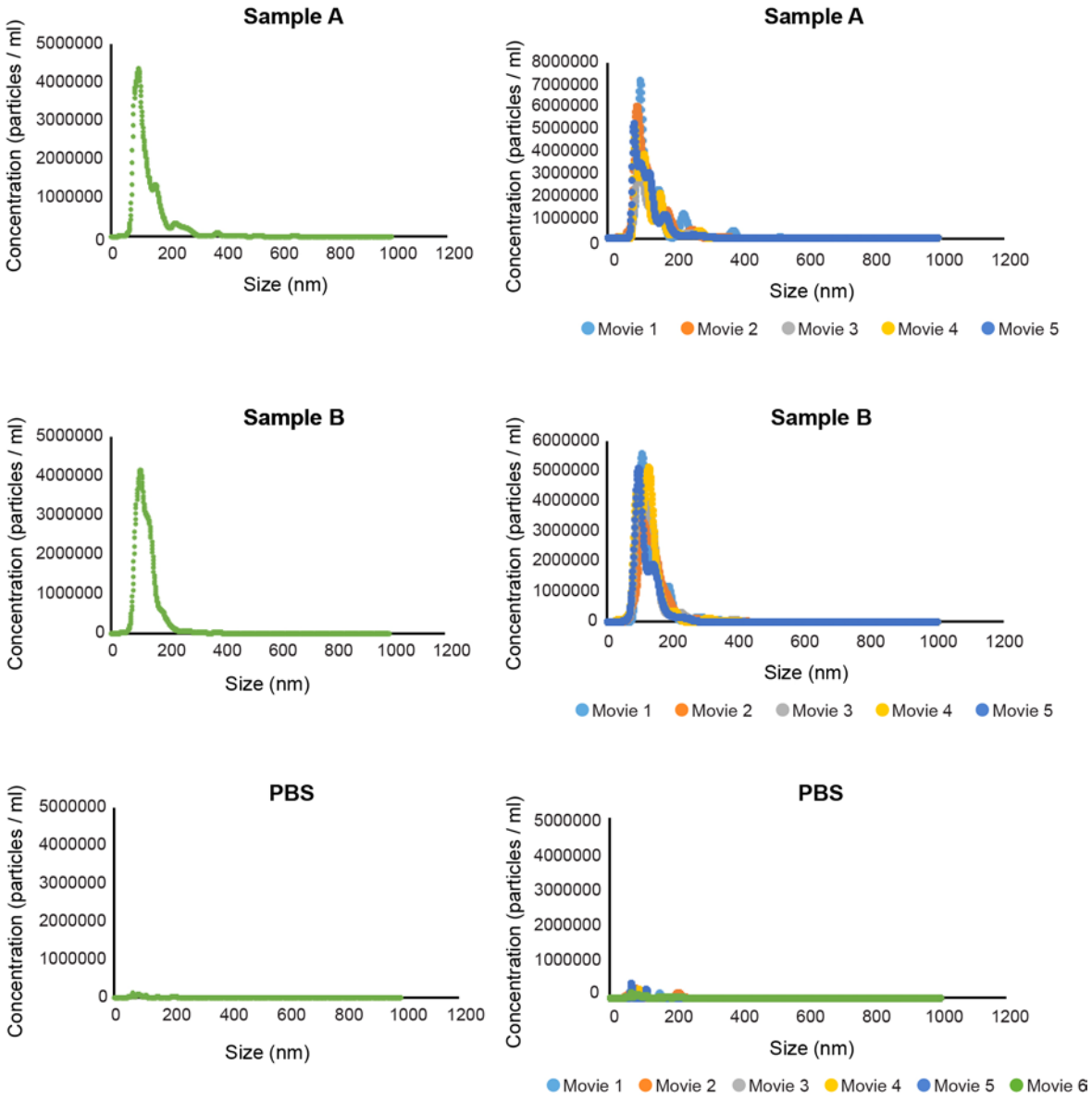
Supp. Figure S8. Related to Figure5.

Percentage of reticulocytes in erythroblasts culture at 2 days, 4 days and 8 days after addition of control medium, iECM or iECM supplemented with serum substitute (BIT), erythropoietin (EPO), L-glutamine (L-Glu), a pool of amino acids (AA), human serum (HS) or all of these components together (Pool). For each graphical, circles indicate the number of independent experiments and error bars show the SEM. *** $p < 0.001$, ** $p < 0.01$, ns: non-significant difference.



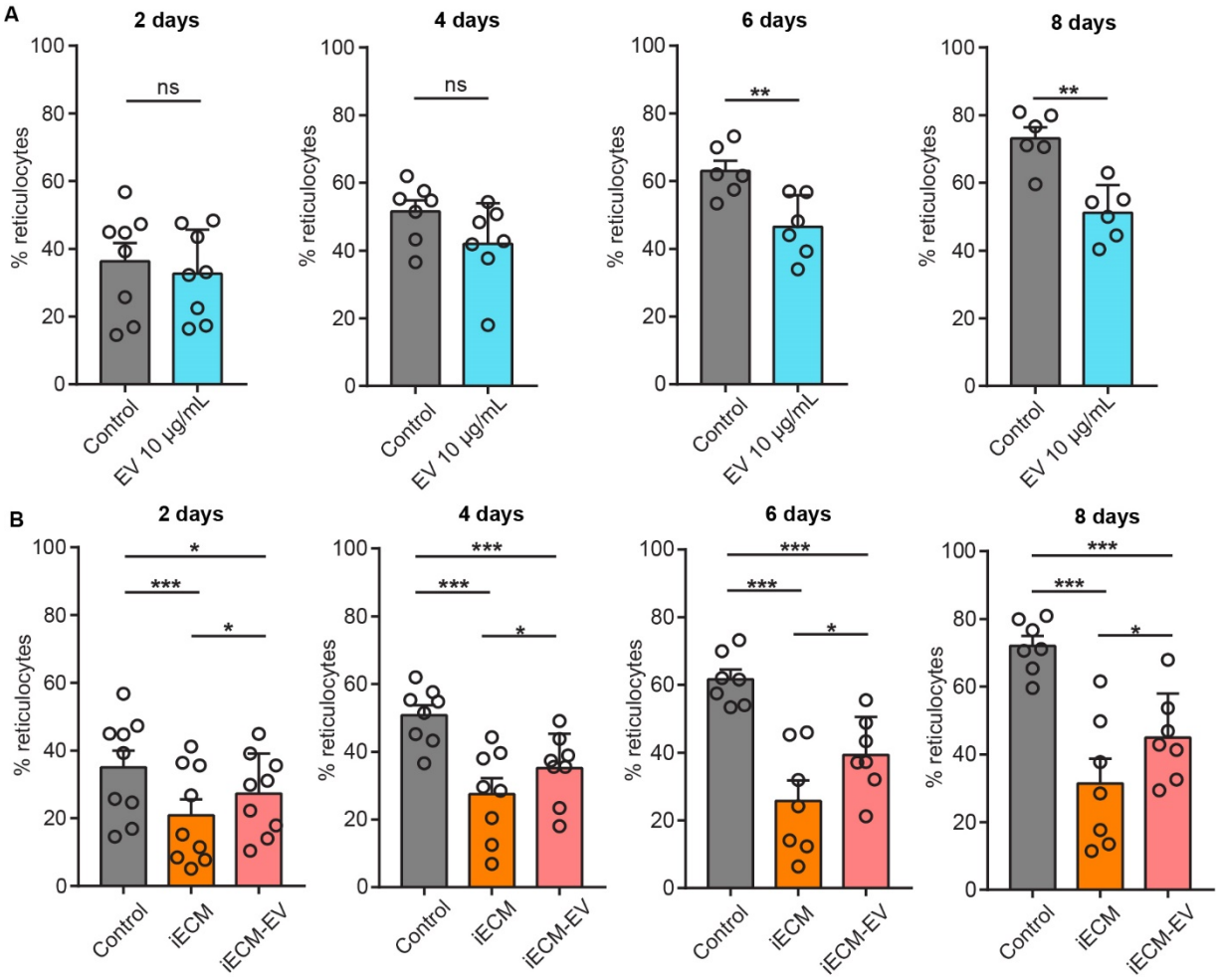
Supp. Figure S9. Related to Figure 6.

Scatter plots showing the gating strategy for EVs observation. (A) Gating strategy on Megamix beads of 900 nm, 500 nm, 300 nm and 100 nm. (B) Gating strategy on purified EVs labeled with GPA-PC7 antibody (upper panel) or with IgG-isotype-PC7 antibody (lower panel).



Supp. Figure S10. Related to Figure 6.

Size distribution analysis by NTA for two biological replicates (A and B) and PBS control. Right: size distribution is displayed for 5 or 6 movies with minimal number of 4865 completed tracks per video and a total of 1498 frames analyzed. Left: size distribution is displayed as an average of 5 or 6 movies.



Supp. Figure S11. Related to Figure 6.

(A) Percentage of reticulocytes in erythroblast culture at 2 days, 4 days, 6 days and 8 days after addition of 10 µg/mL EVs or control medium (Control). (B) Percentage of reticulocytes in erythroblast culture at 2 days, 4 days, 6 days and 8 days after addition of iECM, iECM - EV or control medium (Control). Circles indicate the number of independent experiments and error bars show the SEM. ***p<0.001, ** p < 0.01, * p < 0.05, ns: non-significant difference.

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