Lyse-Reseal Erythrocytes for Transfection of *Plasmodium falciparum*

Gokulapriya Govindarajalu, Zeba Rizvi, Deepak Kumar & Puran Singh Sijwali*

Simple and efficient transfection methods for genetic manipulation of *Plasmodium falciparum* are desirable to identify, characterize and validate the genes with therapeutic potential and better understand parasite biology. Among the available transfection techniques for *P. falciparum*, electroporation-based methods, particularly electroporation of ring-infected RBcs is routinely used. Nonetheless, transfection of *P. falciparum* remains a resource-intensive procedure. Here, we report a simple and economic transfection method for *P. falciparum*, which is termed as the lyse-reseal erythrocytes for transfection (LyRET). It involved lysis of erythrocytes with a hypotonic RBC lysis buffer containing the desired plasmid DNA, followed by resealing by adding a high salt buffer. These DNA-encapsulated lyse-reseal erythrocytes were mixed with *P. falciparum* trophozoite/schizont stages and subjected to selection for the plasmid-encoded drug resistance. In parallel, transfections were also done by the methods utilizing electroporation of DNA into uninfected RBcs and parasite-infected RBcs. The LyRET method successfully transfected 3D7 and D10 strains with different plasmids in 63 of the 65 attempts, with success rate similar to transfection by electroporation of DNA into infected RBcs. The cost effectiveness and comparable efficiency of LyRET method makes it an alternative to the existing transfection methods for *P. falciparum*, particularly in resource-limited settings.

*P. falciparum* causes the most virulent form of malaria and is responsible for the majority of malaria-associated mortality. Lack of a vaccine and continued emergence of drug resistant strains are challenges to malaria control and elimination efforts. Limited understanding of the biology of *P. falciparum* is also a barrier to the identification of targets for development of new chemotherapeutics and diagnostics. The availability of genome sequences of *P. falciparum* and other *Plasmodium* species serves as a treasure to be unlocked for understanding the complex, yet interesting, biology of *Plasmodium*. Manipulation of desired genes or gene products using reverse genetics techniques has been immensely useful in studying model organisms. Early transfection methods for *Plasmodium* utilized electroporation of plasmid DNA into asexual blood stage-infected erythrocytes\(^1\)\(^-\)\(^3\). Attempts have been made to improve transfection efficiency by electroporation of DNA into uninfected erythrocytes followed by infection of these DNA-preloaded erythrocytes by schizont stage parasites (Epreloading method)\(^4\). Electroporation-independent procedures have also been used with varying success\(^5\)\(^-\)\(^9\). Nucleofection of the schizont stage of *P. berghei*, a rodent malaria parasite, greatly enhanced transfection efficiency and decreased selection period\(^10\)\(^,\)\(^11\). Nucleofection has also been adapted for high-throughput transfection of *P. falciparum*\(^12\). However, nucleofection requires expensive proprietary reagents and an electroporation device. Development of transfection procedures for genetic manipulations of *Plasmodium* species and the availability of *Plasmodium* genome sequences have led to several exciting findings on the parasite biology, including the recent reports of genome-wide gene knock outs in *P. falciparum* and *P. berghei\(^13\)\(^,\)\(^14\). Electroporation of DNA into ring stage-infected red blood cells (EiRBC method) remains the most used method in the field at present\(^7\).

Transfection of *P. falciparum* is still considered a resource-intensive procedure. Since *P. falciparum* can take up plasmid DNA present in the erythrocyte cytosol and resealed erythrocytes remain infective to *P. falciparum\(^6\)\(^,\)\(^15\)\(^-\)\(^18\)*, we combined these two properties to develop a transfection procedure. Erythrocytes were lysed with a hypotonic RBC lysis buffer containing the desired plasmid DNA, resealed by adding a high salt buffer, mixed with purified trophozoite/schizont stage parasites, and subjected to drug selection. We have named this method as the lyse-reseal erythrocytes for transfection (LyRET). The success rate of transfection of *P. falciparum* by LyRET was comparable with EiRBCs method and was higher than that of Epreloading method.

CSIR-Centre for Cellular and Molecular Biology, Hyderabad, 500007, TS, India. *email: psjwi@ccmb.res.in
Materials and Methods

*P. falciparum* 3D7 and D10 strains were obtained from the Malaria Research and Reference Reagent Resource Centre (MR4). RPMI 1640 (cat No. 12-115Q) was procured from Lonza. Hypoxanthine (cat No. 11067-030), albumax II (cat No. 11021037), gentamicin (cat No. 15750-060) and blasticidin S HCl (cat no. R210-01) were from Invitrogen. Restriction enzymes and DNA modifying enzymes were from New England Biolabs Inc. and Thermo Fisher Scientific. DNA isolation kits were from Qiagen and MACHEREY-NAGEL. All biochemical were from standard suppliers like Sigma and Serva. All the tissue culture plastic ware was from standard manufacturers such as Corning Inc, Nalgene and Tarsons. All experiments were performed in accordance with relevant guidelines and regulations.

Parasite culture.  Whole blood was collected from human volunteers after obtaining informed consent by venipuncture according to the approved protocols of Institutional Ethics Committee of Centre for Cellular and Molecular Biology (IEC-38/2015 and IEC-38-R3/2019). The blood was centrifuged at 662xg for 5 min at room temperature using a swinging-bucket rotor. Plasma and Buffy coat were carefully removed by aspiration. The RBC pellet was washed twice, each time with 2x packed cell volume (PCV) of the RBC storage medium (RPMI 1640 with 2g/l glucose, 300mg/l glutamine and 25μg/ml gentamicin). The RBCs were stored as 50% hematocrit in RBC storage medium at 4°C until used. RBCs from different donors irrespective of the blood group were used during routine parasite culture and for the majority of transfection experiments, except for transfection experiments with individual blood group RBCs.

The *P. falciparum* strains were grown at 37°C under a mixed gas environment (5% CO₂, 5% O₂ and 90% N₂) in the parasite culture medium (RPMI 1640 with 2g/l sodium bicarbonate, 2g/l glucose, 25μg/ml gentamicin, 300mg/l glutamine, 0.5% albumax II) containing human erythrocytes at 2% hematocrit. Parasites were synchronized by treatment with 5% D-sorbitol when the majority of parasites were at ring stage. For purification of late trophozoite/schizont stages, synchronized ring stage culture (10–15% parasitemia) was grown till majority of the parasites reached late trophozoite/schizont stage. 25–30 ml of the culture was transferred to a 50 ml conical centrifuge tube, 10 ml of 65% nycodenz density gradient solution was carefully layered underneath the culture. The supernatant was centrifuged at 360 × g for 15 min at 25°C using a swinging-bucket rotor (with maximum acceleration and zero deceleration). The interphase containing iRBCs was carefully collected and transferred to a 15 ml conical centrifuge tube. The suspension was centrifuged at 662 × g for 5 min at room temperature using a swinging-bucket rotor, the supernatant was carefully aspirated, the pellet was resuspended in 10 ml parasite culture medium and centrifuged at 662 × g for 5 min at room temperature using a swinging-bucket rotor. The supernatant was discarded and the pellet was washed again with parasite culture medium. The final pellet was suspended in 1 ml parasite culture medium. An aliquot of the purified sample was processed for Giemsa smear to assess purity of the sample.

Transfection plasmids. Three plasmids were used for transfection: pFCEN1, pPfCENv3 and HFDDI. pFCEN1 was a kind gift from Dr. Shiroh Iwanga. It contains human dihydrofolate reductase (hDHFR) cassette for selection of recombinant parasites with WR99210 and GFP cassette for evaluation of recombinant parasites for GFP expression using fluorescence microscopy or western blotting. pPfCENv3 was derived by replacing the GFP coding sequence at AvrII-AflII sites in pPfCENv2 with another GFP coding sequence, which was amplified from the pSTCII-GFP using primers GFPcen-F (ATTACCTAGGAGATCTCAAAATGGGTACC; contains XhoI/AflII sites) and GFPcen-R (ATTACTTAAGCTCGAGTTAGGATCCCTG, contains XhoI/AflII sites) containing the hDHFR cassette for selection of transfected parasites with WR99210. All the three plasmids were prepared using Qiagen or MACHEREY-NAGEL maxi preparation kits according to the manufacturer’s instructions. Plasmid DNA pellets were resuspended in RBC lysis buffer (5mM K₂HPO₄, 1mM ATP, pH 7.4; for LyRET method) or nuclease free water (for electroporation methods).

Preparation of lyse-reseal erythrocytes. The RBC suspension (~200 μl/experiment) was transferred into a 1.5 ml microcentrifuge tube (MCT) and centrifuged at 371 x g for 5 min at 4 °C using a fixed-angle rotor. The supernatant (~100 μl PCV) was washed twice, each time with 1 ml ice cold PBS, and the supernatant was carefully removed. The pellet was resuspended in 100 μl of ice cold RBC lysis buffer (5mM K₂HPO₄, 1mM ATP, pH 7.4) and incubated at 4°C for 1 hour with gentle rotation. The lysed RBC ghost suspension was resealed as has been previously described with minor modifications. The volume of RBC ghost suspension was estimated and appropriate volumes of solution stocks (5mM NaCl, 1mM MgCl₂, 100mM ATP, 100mM GSH) were added to achieve the resealing buffer concentration (150mM NaCl, 5mM MgCl₂, 1mM ATP and 1mM GSH). This suspension was incubated at 37°C for 1 hour with shaking at 55 rpm to allow resealing of RBC ghost. The resealed RBC suspension was washed twice, each time with 10xPCV of RBC storage medium (prewarmed to 37°C). The washed resealed RBC pellet was resuspended in equal volume of RBC storage medium for subsequent use or stored at 4°C till used. The resealed RBCs were termed as the lyse-reseal erythrocytes (LREs).

For increased incorporation of the lysate protein content in resealed RBCs, the RBC ghost suspension was concentrated to 75% of its volume using a 3 kDa cut-off centrifcon before resealing. Concentration was done at 4°C and 2739 × g using a swinging-bucket rotor.

Parasite growth in lyse-reseal erythrocytes. 2.5 ml parasite culture medium containing normal erythrocytes or LREs prepared without or with the concentration step (all at 2% hematocrit) was inoculated with purified trophozoite/schizont stage parasites (1% final parasitemia). The cultures were grown for three consecutive cycles with the change of media and addition of fresh respective erythrocytes at the end of each cycle to maintain
2\% hematocrit. Parasite stages and parasitemia were determined every 24 hours by making Giemsa smears and counting at least 1000 cells. This experiment was set up in triplicates and repeated up to three times.

**Transfection of *P. falciparum* using lyse-reseal erythrocytes.** Detailed step-wise protocol is provided in the Supplementary Information. 100 µl packed cell volume of RBCs was washed twice with 1 ml ice cold PBS, lysed with ice cold RBC lysis buffer containing 100 µg plasmid DNA, and ressealed as mentioned above in the "Preparation of lyse-reseal erythrocytes" section. LREs containing DNA were resuspended in 5 ml parasite culture medium (at 2\% hematocrit), inoculated with purified trophozoite/schizont stages to achieve 2–3\% parasitemia (day 0), and cultured under standard conditions. On the following day (day 1), the culture was fed with fresh parasite culture medium. On day 2, the culture was expanded to adjust parasitemia to 4–5\%, and hematocrit was maintained at 2\% by adding fresh normal RBCs. On day 3, selection for recombinant parasites was started by adding appropriate drugs in the parasite culture medium (blasticidin: 1µg/ml for pPICENv3; WR99210: 0.5–1.0 nM for HFDDI and pFCEN1). Cultures were grown under drug pressure for 5 consecutive cycles to eliminate non-transfected parasites, followed by in the absence of drug for 3 cycles to reduce stress to transfected parasites, and thereafter in the presence of drug. The culture medium was changed every day for the first week, followed by on alternate days, and 50 µl suspension of fresh normal RBCs was added to the culture once a week to replenish old and lysed RBCs. The cultures were routinely monitored for parasites by observing Giemsa smears. Upon emergence of recombinant parasites, the cultures were expanded and processed for downstream experiments like preparation of frozen stocks and evaluation of recombinant parasites.

pPICENv3 was also transfected into RBCs of different blood groups (O+, A+, B+ and AB+) as has been described above. Briefly, 100 µg plasmid DNA was used to prepare LREs of each RBC group, trophozoite/schizont stage parasites were purified from a synchronized culture of *P. falciparum* 3D7 maintained in O blood group RBCs, and the RBCs of respective blood groups were used whenever fresh normal RBCs needed to be added to the culture.

**Transfection by electroporation of infected-RBCs.** Transfection of ring-stage-infected RBCs (EiRBC method) was performed as has been previously described with minor modifications. Briefly, 5 ml culture of early ring stage-infected RBCs (5–10\% parasitemia) was used for one transfection. The culture was centrifuged at 371 \times g for 5 min at room temperature using a fixed-angle rotor, the pellet (~100 µl packed cell volume) was washed with cytomix (10 mM K2HPO4-KH2PO4, 120 mM KCl, 0.15 mM CaCl2.2H2O, 25 mM HEPES, 2 mM EGTA, 5 mM MgCl2.6H2O, pH 7.6) and then suspended in cytomix containing 50 µg plasmid DNA (final volume: 420 µl). The suspension was transferred to a chilled 0.2 cm cuvette and pulsed (at 0.31 kV, 960 µF and infinite Ω) using the Bio-Rad gene pulser Xcell™. The electroporated sample was immediately transferred to a flask with 5 ml parasite culture medium, and grown under standard conditions. 3–4 hours post-transfection, the culture medium was changed with fresh parasite culture medium (day 0). On day 1, the culture was expanded to 10 ml with fresh RBCs. Selection was started with the addition of appropriate drug to the culture on day 2. The parasite culture medium was changed every day for the first week, followed by on alternate days, and 50 µl fresh RBC suspension was added to the culture once a week to replenish old and lysed RBCs. The cultures were maintained thereafter as described above for the LyRET method.

pPICENv3 was also transfected into RBCs of O blood group as has been described above. Briefly, early ring-stage infected RBCs of O blood group were electroporated with 50 µg plasmid DNA and the RBCs of same blood group were used whenever fresh normal RBCs needed to be added to the culture.

**Transfection by electroporation of DNA into uninfected RBCs.** Electroporation of DNA into uninfected RBCs (Epreloading method) was performed as has been described earlier with minor modifications. Briefly, uninfected RBCs (100 µl packed cell volume/transfection) were washed with cytomix, and then resuspended in cytomix containing 50 µg plasmid DNA (final volume: 420 µl). The suspension was transferred to a 0.2 cm cuvette and electroporated as described above for iRBCs method. The electroporated sample was transferred to a 15 ml conical centrifuge tube with 5 ml parasite culture medium, centrifuged at 662 \times g for 5 min at room temperature using a swinging-bucket rotor. The pellet of DNA-preloaded RBCs was resuspended in 5 ml parasite culture medium, mixed with purified trophozoite/schizont stage parasites (2–3\% final parasitemia), and grown under standard conditions (day 0). From day 1 onward, the culture was maintained as described for the LyRET method.

**Assessment of recombinant parasites.** The pPICENv3 and pFCEN1 transfected parasites were assessed for GFP expression using live cell fluorescence microscopy, and the HFDDI transfected parasites were assessed for the presence of plasmid DNA by PCR. For microscopy, a small aliquot of the culture was washed twice with PBS, stained with Hoechst (10 µg/ml), and immobilized on a poly L-Lysine coated slide for 20 minutes. Unbound cells were washed off with PBS and the slide was covered with a coverslip. The slide was observed under the 100x objective of ZEISS Axioimager microscope. Images were taken (Zeiss AxioCam HRm) and analysed with the Axiovision software. For PCR, the HFDDI transfected parasites were isolated from a 10 ml asynchronous culture (10–15% parasitemia) by saponin lysis. The parasite pellet was processed for genomic DNA isolation using the Puregene blood kit (Qiagen) as instructed by the manufacturer. Genomic DNA was used as a template for amplification of the *P. yoelii* α-tubulin region of HFDDI by PCR (primers: PyaTb5U-F: AGGGACCGGTGAAAAGCCCTAAATGC, g for 5 min at room temperature using a fixed-angle rotor, the pellet (~100 µl packed cell volume) was washed with cytomix (10 mM K2HPO4-KH2PO4, 120 mM KCl, 0.15 mM CaCl2.2H2O, 25 mM HEPES, 2 mM EGTA, 5 mM MgCl2.6H2O, pH 7.6) and then suspended in cytomix containing 50 µg plasmid DNA (final volume: 420 µl). The suspension was transferred to a chilled 0.2 cm cuvette and pulsed (at 0.31 kV, 960 µF and infinite Ω) using the Bio-Rad gene pulser Xcell™. The electroporated sample was immediately transferred to a flask with 5 ml parasite culture medium, and grown under standard conditions. 3–4 hours post-transfection, the culture medium was changed with fresh parasite culture medium (day 0). On day 1, the culture was expanded to 10 ml with fresh RBCs. Selection was started with the addition of appropriate drug to the culture on day 2. The parasite culture medium was changed every day for the first week, followed by on alternate days, and 50 µl fresh RBC suspension was added to the culture once a week to replenish old and lysed RBCs. The cultures were maintained thereafter as described above for the LyRET method.

pPICENv3 was also transfected into RBCs of O blood group as has been described above. Briefly, early ring-stage infected RBCs of O blood group were electroporated with 50 µg plasmid DNA and the RBCs of same blood group were used whenever fresh normal RBCs needed to be added to the culture.
In transfections by both LyRET and EiRBC methods, the emergence of resistant parasites was significantly delayed in case of HFDDI and pFCEN1 compared to those with pPfCENv3 (Table 1). This delay could be due to difference in the expression levels of selection markers (BSD in pPfCENv3 versus hDHFR in HFDDI and pFCEN1), as the selection marker genes are under different regulatory sequences (Supplementary Information). Whether this delay is also attributed to the drug used for selection remains to be tested. One can test this using pFCEN1, as the selection marker genes are under different regulatory sequences (Supplementary Information).
with HFDDI and pFCEN1 plasmids. We speculate that this difference may be due to the potential of EiRBC method to electroporate some DNA molecules directly into the parasite, whereas parasites have to take up DNA from the RBC cytosol in case of LyRET. The different mode of introducing DNA by LyRET and EiRBC methods together with the intrinsic nature of HFDDI and pFCEN1 plasmids might have been responsible for the overall delay.

Most of the transfection experiments with *P. falciparum* require 50–100 µg plasmid DNA/transfection. Hence, we tested a range of plasmid DNA amounts (1 to 100 µg/transfection) to determine the minimum plasmid DNA amount for successful transfection by the LyRET method. Drug resistant parasites emerged in 12–24 days with 2.5–100 µg plasmid DNA/transfection (Table 2), and the parasites from these experiments showed GFP
fluorescence in live cell microscopy (data not shown). Transfections without any plasmid DNA or with 1.0 μg plasmid DNA/transfection yielded unstable drug resistant parasites, which did not show GFP and were eliminated at higher selection pressure, indicating that these were untransfected parasites (Table 2). 2.5 μg is the minimum amount of plasmid DNA for successful transfection by the LyRET method. However, resistant parasites emerged almost 12 days earlier in transfections with ≥40 μg plasmid DNA as compared to transfections with 2.5 μg plasmid DNA. Hence, 40 μg of plasmid DNA appears to be optimum amount for transfection by the LyRET method.

As *P. falciparum* strains have been shown to invade RBCs of different blood groups with different efficiency in *in vitro* conditions,[25] we assessed the LyRET method for transfection of RBCs of different blood groups. Recombinant parasites were obtained with RBCs of all the blood groups, which, however, differed in days required to reach 1–2% parasitemia (Table 3). Transfections of blood group O by the LyRET method was the most efficient in terms of the number of days required to reach 1–2% parasitemia and %GFP population, and it was comparable with the EiRBC method for the same blood group (Table 3). Live fluorescence microscopy of

**Table 2.** Optimization of plasmid DNA amount for transfection of *P. falciparum* 3D7 by the LyRET method. Each transfection experiment was independently performed. The days to emergence of resistant parasites is mean with SD of the days required for emergence of resistant parasites in successful experiments.

| μg of pPfCENv3/ transfection | No. of successful transfections/total No. of transfections done | Days to emergence of resistant parasites |
|-------------------------------|---------------------------------------------------------------|----------------------------------------|
| 0                             | 0/2                                                           | —                                      |
| 1                             | 0/2                                                           | —                                      |
| 2.5                           | 2/2                                                           | 24 ± 1.4                               |
| 5                             | 2/2                                                           | 23 ± 0                                 |
| 10                            | 6/6                                                           | 17 ± 4.7                               |
| 20                            | 4/4                                                           | 16 ± 1.2                               |
| 40                            | 4/4                                                           | 12.5 ± 1.9                             |
| 80                            | 3/3                                                           | 13 ± 2                                 |
| 100                           | 4/4                                                           | 12 ± 2                                 |
Table 3. Transfection of P. falciparum 3D7 with pPfCENv3 using RBCs of different blood groups by the LyRET method. Each transfection experiment was independently performed. The days to reach 1–2% parasitemia is mean with SD of the days required for emergence of resistant parasites in successful experiments. The %GFP positive cells was determined by FACS and is mean with SD of GFP-Hoechst positive cells in all the successful experiments. *NA indicates “Not Attempted”.

| Blood groups | No. of successful transfections/total No. of transfections done | Days to reach 1–2% parasitemia | %GFP positive cells | No. of successful transfections/total No. of transfections done | Days to reach 1–2% parasitemia | %GFP positive cells |
|--------------|-------------------------------------------------|--------------------------------|-------------------|-------------------------------------------------|--------------------------------|-------------------|
| O+           | 4/4                                             | 20.0 ± 0.0                     | 53.1 ± 8.5        | 4/4                                              | 19.5 ± 0.7                     | 49.2 ± 6.6        |
| A+           | 4/4                                             | 35.0 ± 9.9                     | 32.2 ± 3.1        | *NA                                             | NA                             | NA                |
| B+           | 4/4                                             | 20.5 ± 0.7                     | 48.3 ± 24.5       | NA                                              | NA                             | NA                |
| AB+          | 4/4                                             | 34.0 ± 11.3                    | 34.3 ± 11.7       | NA                                              | NA                             | NA                |

References

1. Goonewardene, R. et al. Transfection of the malaria parasite and expression of firefly luciferase. *Proceedings of the National Academy of Sciences* 90, 5234–5236 (1993).
2. van Dijk, M. R., Waters, A. P. & Janse, C. J. Stable transfection of malaria parasite blood stages. *Science* 268, 1358–1362 (1995).
3. Wu, Y., Sfiri, C. D., Lai, H. H., Su, X. Z. & Wellems, T. E. Transfection of *Plasmodium falciparum* within human red blood cells. *Proc Natl Acad Sci USA* 92, 973–977 (1995).
4. Wu, Y., Kirkman, L. A. & Wellems, T. E. Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmons that confer resistance to pyrimethamine. *Proc Natl Acad Sci USA* 93, 1130–1134 (1996).
5. Fidock, D. A. & Wellems, T. E. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. *Proc Natl Acad Sci USA* 94, 10931–10936 (1997).
6. Deitsch, K., Driskill, C. & Wellems, T. Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes. *Nucleic Acids Res* 29, 850–853 (2001).
7. Mamoun, C. B. et al. Transfer of genes into *Plasmodium falciparum* by polyamidoamine dendrimers. *Mol Biochem Parasitol* 103, 117–121 (1999).
8. Gopalakrishnan, A. M., Kundu, A. K., Mandal, T. K. & Kumar, N. Novel nanosomes for gene delivery to *Plasmodium falciparum*-infected red blood cells. *Sci Rep* 3, 1534, https://doi.org/10.1038/srep01534 (2013).
9. Fotoran, W. L., Santangelo, R., de Miranda, B. N. M., Irvine, D. I. & Wunderlich, G. DNA-Loaded Cationic Liposomes Efficiently Function as a Vaccine against Malarial Proteins. *Mol Ther Methods Clin Dev* 7, 1–10, https://doi.org/10.1016/j.omtm.2017.08.004 (2017).
10. Janse, C. J. et al. High efficiency transfection of *Plasmodium berghei* facilitates novel selection procedures. *Molecular and Biochemical Parasitology* 145, 60–70 (2006).
11. Janse, C. J., Ramesar, J. & Waters, A. P. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite *Plasmodium berghei*. *Nature Protocols* 1, 346–356 (2006).
12. Caro, F., Miller, M. G. & DeRisi, J. L. Plate-based transfection and culturing technique for genetic manipulation of *Plasmodium falciparum*. *Malaria journal* 11, 22–22, https://doi.org/10.1186/1475-2875-11-22 (2012).
13. Bushell, E. et al. Functional Profiling of a Plasmodium Genome Reveals an Abundance of Essential Genes. *Cell* 170, 260–272 (2017).
14. Zhang, M. et al. Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. *Science* 360, https://doi.org/10.1126/science.aap7847 (2018).
15. Murphy, S. C. et al. Erythrocyte G protein as a novel target for malarial chemotherapy. *PLoS Med* 3, e528, https://doi.org/10.1371/journal.pmed.0030528 (2006).
16. Frankland, S. et al. Delivery of the malaria virulence protein PfEMP1 to the erythrocyte surface requires cholesterol-rich domains. *Eukaryot Cell* 5, 849–860 (2006).
17. Chandramohandas, R. et al. Apicomplexan parasites co-opt host calpains to facilitate their escape from infected cells. *Science* 324, 794–797 (2009).
18. Abu Bakar, N., Klonis, N., Hansen, E., Chan, C. & Tilley, L. Digestive- vacuole genesis and endocytic processes in the early intraerythrocytic stages of *Plasmodium falciparum*. *J Cell Sci* 123, 441–450 (2010).
19. Trager, W. & Jensen, J. R. Human malaria parasites in continuous culture. *Science* 193, 673–675 (1976).
20. Lambros, C. & Vanderberg, J. P. Synchronization of Plasmodium falciparum erythrocytic stages in culture. J Parasitol 65, 418–420 (1979).

21. Iwanaga, S., Kato, T., Kaneko, I. & Yuda, M. Centromere Plasmid: A New Genetic Tool for the Study of Plasmodium falciparum. PLOS ONE 7, e33326, https://doi.org/10.1371/journal.pone.0033326 (2012).

22. Sijwali, P. S. & Rosenthal, P. J. Functional evaluation of Plasmodium export signals in Plasmodium berghei suggests multiple modes of protein export. PLoS One 5, e10227, https://doi.org/10.1371/journal.pone.0010227 (2010).

23. Singhal, N., Atul, Mastan, B. S., Kumar, K. A. & Sijwali, P. S. Genetic ablation of plasmoDJ1, a multi-activity enzyme, attenuates parasite virulence and reduces oocyst production. Biochem J 461, 189–203 (2014).

24. Sijwali, P. S., Koo, J., Singh, N. & Rosenthal, P. J. Gene disruptions demonstrate independent roles for the four falcipain cysteine proteases of Plasmodium falciparum. Mol Biochem Parasitol 150, 96–106 (2006).

25. Theron, M., Cross, N., Cawkill, P., Bustamante, L. Y. & Rayner, J. C. An in vitro erythrocyte preference assay reveals that Plasmodium falciparum parasites prefer Type O over Type A erythrocytes. Scientific Reports 8, 8133, https://doi.org/10.1038/s41598-018-26559-2 (2018).

Acknowledgements
This study and the salary of GG were supported with funds (BT/COE/34/SP15138/2015) from the Department of Biotechnology, India. ZR and DK are recipients of fellowships from the Department of Biotechnology, India.

Author contributions
G.G. designed and performed experiments, generated and interpreted data, and helped in writing the manuscript. Z.R. performed transfection experiments with 3D7 and D10 strains, interpreted data, and helped in writing the manuscript. D.K. performed transfection experiments with D10 strain and interpreted data. P.S.S. conceived the idea and designed experiments, discussed and interpreted data, and wrote the manuscript with other authors.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-019-56513-9.

Correspondence and requests for materials should be addressed to P.S.S.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019