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Generation of a new DiCre expressing parasite strain for functional characterization of *Plasmodium falciparum* genes in blood stages

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Conditional regulation is a highly beneficial system for studying the function of essential genes in *Plasmodium falciparum* and dimerizable Cre recombinase (DiCre) is a recently adapted conditional regulation system suitable for this purpose. In the DiCre system, two inactive fragments of Cre are reconstituted to form a functionally active enzyme in the presence of rapamycin. Different loci have been targeted to generate parasite lines that express the DiCre enzyme. Here, we have used marker-free CRISPR-Cas9 gene editing to integrate the DiCre cassette in a redundant *cg6* locus. We have shown the utility of the newly generated Δcg6DC4 parasites in mediating robust, rapid, and highly specific excision of exogenously encoded *gfp* sequence. The Δcg6DC4 parasites are also capable of conditional excision of an endogenous parasite gene, PF3D7_1246000. Conditional deletion of PF3D7_1246000 did not cause any inhibition in the asexual proliferation of the parasites. Furthermore, the health and morphology of the mutant parasites were comparable to that of the control parasites in Giemsa smears. The availability of another stable DiCre parasite strain competent for conditional excision of target genes will expedite functional characterization and validation of novel drug and vaccine targets against malaria.

Keywords Plasmodium, Dimerizable cre recombinase, LoxP, CRISPR-Cas9, Rapamycin, Conditional regulation

Malaria is one of the top infectious diseases of developing nations accounting for nearly 249 million cases and more than 600,000 deaths worldwide[1]. Concerted efforts in vector control, disease surveillance, and treatment strategies have resulted in an appreciable reduction in malaria-related deaths in the last decade[2]. However, due to multiple factors including the development of drug resistance by the malaria parasite to artemisinin and other partner drugs, further decline in malaria cases has become stagnant worldwide[3,4]. Therefore, new targets must be identified for developing drugs and vaccines against malaria so that the progress made in the last few decades against the disease is not in vain. Identification of novel drug and vaccine targets requires understanding the functional role of essential parasite genes. To elucidate the function of essential genes at different stages of parasite development various conditional knock-down and knock-out strategies are available that work at either gene, transcript, or protein level[5]. For conditional regulation of protein expression in the parasite, degradation domains based on *E. coli* dihydrofolate reductase[6] and FK-506-binding protein are available[7]. In these conditional knock-down systems the fusion protein is stabilized in the presence of a small compound whereas in the absence of the compound, the fusion protein is unstable and is degraded through proteasomal degradation pathway[6,7]. Knock sideways is another technique through which the native localization of a target protein can be conditionally changed using a 'mislocalizer' protein[8]. Another strategy called auxin inducible degron (AID) requires expression of the target protein with an AID tag in a system that co-expresses TIR1, a plant-specific auxin binding domain[9,10]. Proteasomal degradation of AID-tagged target protein occurs in the presence of auxin in the background of TIR1 expressing parasites[11]. The AID system works well in *Toxoplasma gondii*[12,13], a related Apicomplexan parasite however, it has not found much usage in *P. falciparum*. Conditional

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Knock-down at the transcript level is achieved by using anhydrotetracyclic-regulated transactivators consisting of TetR fused to an activation domain[14], transactivation domains encoded within transcription factors belonging to Apicomplexan AP2 family[15], fusion of TetR with effectors such as DOZI (Development of Zygote Inhibited) in combination with TetR aptamers[16], and glmS ribozyme sequence[17].

Conditional regulation at the DNA level is a powerful strategy to understand parasite gene function. A dimerizable Cre recombinase (DiCre) is one such conditional knock-out tool that has been adapted for studying essential malaria parasite genes. Cre recombinase is a bacteriophage enzyme that recognizes a small DNA sequence of 34 bp called loxP site and mediates excision of intervening region flanked by two loxP sites oriented in the same direction[18]. Conditional regulation of Cre recombinase has provided unprecedented opportunities for the study of essential genes that cannot be otherwise studied by direct knock-out methodology. DiCre system consists of two separate inactive fragments of Cre recombinase enzyme, Cre59 and Cre60 that are appended to the FK506-binding protein (FKBP) and the ligand binding domain of FKBP-rapamycin associated protein (FRB)[19,20]. In the presence of rapamycin, FKBP and FRB interact with each other reconstituting a functionally active Cre recombinase[21–23]. Functionally active Cre recombinase specifically recognizes the loxP sites and mediates their recombination leading to the excision of the intervening DNA sequence. DiCre is a highly specific, robust, and efficient conditional regulation system that has been adopted as a method of choice for studying essential parasite genes.

Usage of the DiCre system necessitates the integration of loxP sites at the target locus either in the native intronic sequences or embedded in artificial introns[24] in a parasite that expresses the inactive fragments of Cre recombinase. DiCre expression cassette can be either integrated into the parasite genome to generate stable lines or expressed transiently through an episomal plasmid[25–27]. Collins et al. integrated the DiCre cassette at the 3' utr of *sera5*[27]. SERA5 is abundantly expressed in schizonts and is thought to be involved in the egress of merozoites/invasion of RBCs[28–30]. A mutant parasite containing *attB* site at *cg6* locus[31] has been previously used to generate a DiCre expressing parasite strain[25]. DiCre expression cassette has also been integrated independently in other loci such as *rh3* and *pfs47*[32,33]. To further broaden the utility of the DiCre conditional regulation system for the study of malaria parasite genes in asexual blood stages, here we have integrated the DiCre cassette at the wild type *cg6* locus, a well-characterized redundant locus for the malaria parasite life cycle[31,34]. We have shown the utility of the newly developed conditional system for the regulation of exogenously expressed GFP protein. Importantly, we have demonstrated the utility of this system for the study of endogenous parasite genes. Using this system here we have shown robust deletion of a previously deemed essential parasite gene, PF3D7_1246000. Unexpectedly, disruption of PF3D7_1246000 did not result in any perceptible effect in the asexual proliferation of the parasite under in vitro growth conditions.

Results

Generation of a stable DiCre expressing parasite strain

To generate a parasite strain that stably expresses dimerizable Cre recombinase (DiCre), we targeted the *cg6* locus, a redundant gene for asexual proliferation and subsequent stages of parasite development[31,34]. The 5' and 3' homology regions corresponding to the DNA sequences of *cg6* were cloned in pBSp230pDiCre plasmid[35] flanking the DiCre cassette to generate the final plasmid, pBS-cg6-DiCre (Fig. 1a). Primer pairs: 5HRcg6F/5HRcg6R and 3HRcg6F/3HRcg6R were used to amplify 5' and 3' HR of 368 bp and 330 bp, respectively. Each cloning step was verified by a diagnostic PCR with gene-specific primers followed by DNA sequencing (data not shown). A second plasmid, pL6-Cas9-DHODH, was used to clone a guide region of 20 nucleotides corresponding to the target *cg6* locus using In-Fusion. The sequence of the guide in the final plasmid, pL6-cas9-DHODH-cg6G was verified using Sanger DNA sequencing (data not shown). The two plasmids were co-transfected in the ring stage of WT parasites using conditions reported earlier[36,37]. After 24–48 h of transfection, the parasites were selected with DSM267[38]. DSM267-resistant parasites were visible by Giemsa smear after 3 weeks of transfection. Limiting dilution was used to obtain clonal parasites. Two clones of transgenic parasites ($\Delta cg6^{DicreC4}$ and $\Delta cg6^{DicreC8}$) were used to verify *cg6* disruption via integration of the DiCre cassette. Two different primer pairs: F1/R1 and F2/R3 were designed for specific amplification in $\Delta cg6^{Dicre}$ clonal parasites while F1/R2 was for amplification in only the WT parasites (Fig. 1b). The primer pairs, F1/R1 and F2/R3 were used to confirm the 5' and 3' integration of DiCre cassette at *cg6* locus, respectively and resulted in amplicons of desired sizes, 1842 bp and 2243 bp, respectively (Fig. 1b). Primer pair F1/R2 resulted in specific amplification of desired size (686 bp) only in the WT parasites since the R2 oligo corresponds to a region deleted in the $\Delta cg6^{Dicre}$ clonal parasites. The diagnostic PCR confirms successful disruption of the *cg6* locus and concomitant integration of the DiCre cassette. The DNA sequence of all the primers is provided in Table 1 (Supplementary File). To evaluate any undesired effects of DiCre integration in the *cg6* locus, the growth profile of $\Delta cg6^{DicreC4}$ and $\Delta cg6^{DicreC8}$ was compared to the WT parasites. All three parasite lines were sorbitol-synchronized and seeded at 0.2% parasitemia. The increase in parasitemia was monitored every 24 h for 5 days through SYBR Green I assay[39,40]. The growth of $\Delta cg6^{DicreC4}$ was similar to the WT parasites ($p > 0.05$, Fig. 1c) while $\Delta cg6^{DicreC8}$ showed a modest increase in growth ($p < 0.05$, Fig. 1c) compared with the WT. These results confirm that the *cg6* locus[31,34] is redundant for the asexual replication of parasites. We selected the $\Delta cg6^{DicreC4}$ parasite clone for all further experiments since it showed the same growth as the WT parasite and have referred to it as Δcg6DC4 further on.

DiCre expressing parasites show robust, rapamycin dependent excision of loxP flanked gene sequences

After confirming the integration of the DiCre cassette in the *cg6* locus, we sought to evaluate the potential of Δcg6DC4 parasites for conditional excision of genes floxed with loxP sites. To test rapamycin-induced excision of loxP-targeted genes, the Δcg6DC4 parasites were transfected with a plasmid, pHH3-SP-loxPint-GFP-loxP[35], containing the *gfp* gene with adjacent loxP sites (Fig. 2a). The transfected parasites were selected with blasticidin

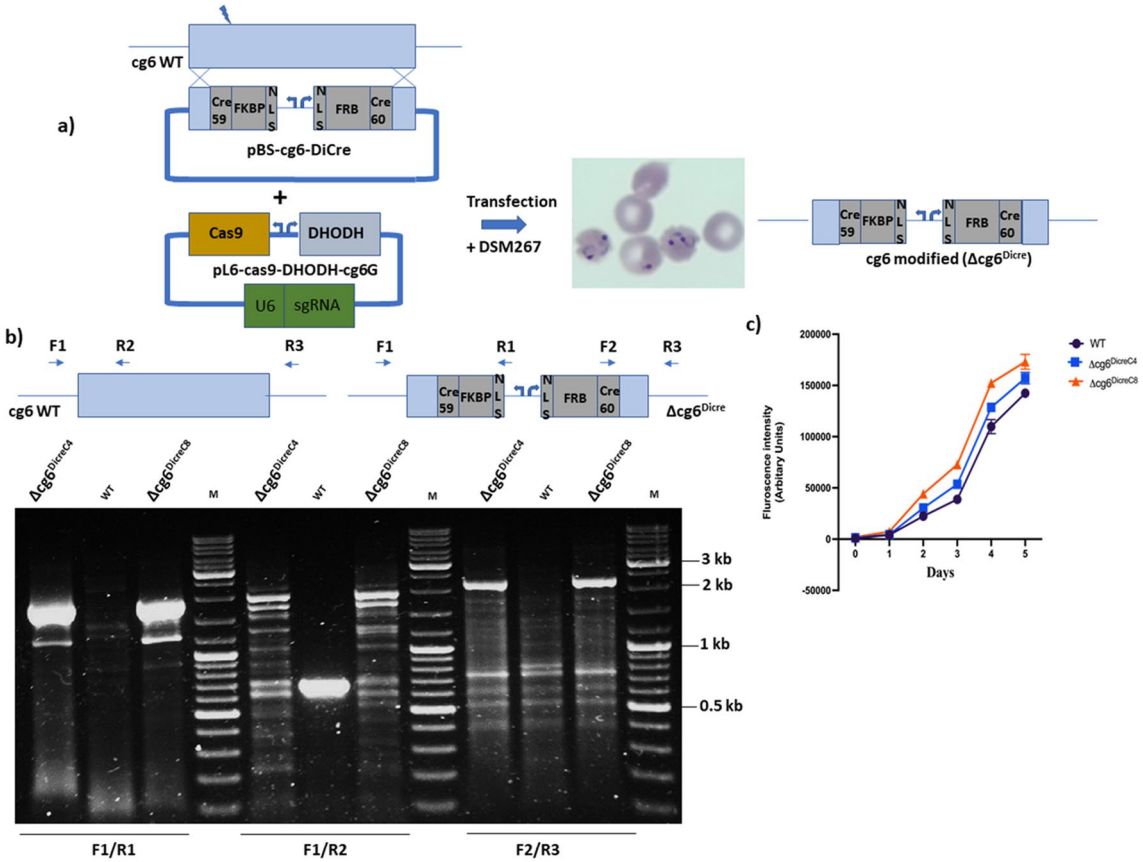

Figure 1. Schematic and verification for desired integration of dimerizable Cre (DiCre) recombinase in*cg6*locus. (**a**) Integration of DiCre expression cassette in *cg6* through marker-free gene editing. The plasmids pBS-cg6-DiCre encoding DiCre cassette and pL6-cas9-DHODH-cg6G encoding Cas9 endonuclease and guide RNA were co-transfected in ring stage of wild type (WT) parasites. Transfected parasites were positively selected with DSM267. The Cas9 mediated double strand breaks at the target site were repaired using the rescue plasmid, pBS-cg6-DiCre. This results in the integration of the DiCre cassette in the *cg6* locus concomitant with its disruption (Δcg6^{Dicre}). The DiCre cassette is constituted of two Cre fragments: Cre59 and Cre60 conjugated to FKBP and FRB, respectively. In the presence of rapamycin FKBP and FRB interact with each other resulting in reconstitution of functional Cre recombinase. (**b**) Two clonal parasites of Δcg6^{Dicre} (clone 4 & clone 8) were used for verifying the integration of DiCre in *cg6* through a diagnostic PCR with gene specific primer sets. The primer sets F1/R1 and F2/R3 amplify 1842 bp and 2243 bp region only in Δcg6^{Dicre} clonal parasites and not in the WT parasites. Primer pair F1/R2 amplifies 686 bp region only in the WT and not in the Δcg6^{Dicre} clonal parasites. M- GeneRuler DNA Ladder Mix. (**c**) Asexual proliferation of Δcg6^{Dicre} clone 4 is similar to the WT parasites. The Δcg6^{DicreC4} and Δcg6^{DicreC8} were used for comparing asexual replication with the WT parasites using SYBR Green I assay. The fluorescence intensity (arbitrary units) and the number of days is plotted on the Y and X-axis, respectively. The error bars represent Mean ± SD (Mean ± standard deviation) in triplicate samples in a single experiment. The graph is plotted using GraphPad Prism 9. Statistical significance between the growth of Δcg6^{Dicre} parasites compared to the WT was calculated using multiple paired t-tests.

S-deaminase (BSD). Before testing rapamycin inducible excision of *gfp*, we verified the presence of the plasmid in the drug-resistant parasites. To this end, a diagnostic PCR was set up using a primer set, MSP3F/hrp2R, specific for the *gfp* cassette (Fig. 2a). With MSP3F/hrp2R, an amplicon of the desired size (1471 bp) was obtained only in the drug-resistant parasites and not in Δcg6DC4 parasites (Fig. 2a). Primer pair, F1/R1 that is specific for the modified *cg6* locus resulted in an amplicon of desired size (1842 bp) in both the parasites (Fig. 2a). We further tested the expression of GFP protein in Δcg6DC4:pHH3GFP parasites using Western blot analysis. Parasite lysates prepared from synchronized trophozoite stage of Δcg6DC4:pHH3GFP and Δcg6DC4 parasites were separated on SDS-PAGE followed by Western blot with anti-GFP antibodies (anti-GFP Ab). Band of desired size (~ 26 kDa) corresponding to full-length GFP was specifically observed only in the Δcg6DC4:pHH3GFP parasites and not in the Δcg6DC4 parasites (Fig. 2b). As a loading control, a parallel blot was probed with anti-beta-actin antibodies (anti-β-actin Ab) (Fig. 2b). Taken together, the PCR and the Western blot results confirm the presence of the plasmid and expression of GFP in Δcg6DC4:pHH3GFP parasites.

Next, we tested rapamycin-mediated excision of loxP-flanked *gfp* in Δcg6DC4:pHH3GFP parasites. For standardization of rapamycin treatment conditions, the Δcg6DC4:pHH3GFP parasites were sorbitol

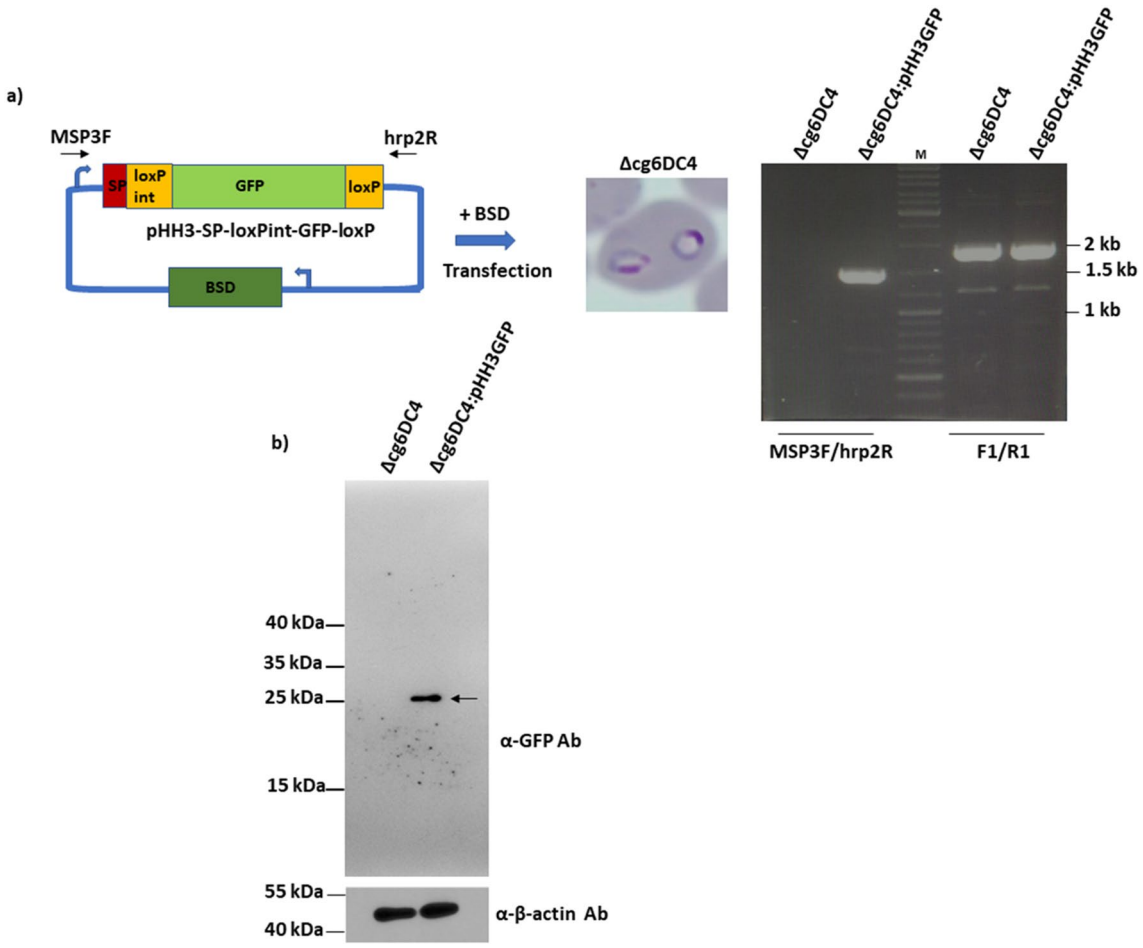

Figure 2. Exogenous expression of green fluorescent protein (GFP) in Δcg6DC4 parasites. (**a**) The plasmid pHH3-SP-loxPint-GFP-loxP containing the *gfp* gene with adjacent loxP sequences was transfected in clone 4 of Δcg6^{Dicre} (Δcg6DC4) parasites and selected with blasticidin S-deaminase (BSD). Presence of the plasmid was verified using MSP3F/hrp2R primers. An amplicon of 1471 bp was obtained only in Δcg6DC4:pHH3GFP parasites containing episomal copies of pHH3-SP-loxPint-GFP-loxP. The primers F1/R1, specific for amplification of the disrupted *cg6* locus result in an amplicon of 1842 bp in both Δcg6DC4:pHH3GFP and Δcg6DC4 parasites. SP- 110 bp signal-peptide of *msp3* gene. (**b**) Verification of GFP expression in Δcg6DC4:pHH3GFP parasites. Parasite lysates from Δcg6DC4:pHH3GFP and Δcg6DC4 were separated on SDS-PAGE and probed with anti-GFP antibodies. A band corresponding to the desired size of GFP protein (shown with a black arrow) was detected only in the Δcg6DC4:pHH3GFP parasite lysate. Anti-β-actin antibodies were used as loading control for the parasite material.

synchronized followed by treatment with two different concentrations of rapamycin (100 nM and 200 nM) for 4 h and 8 h, respectively. All four treatment conditions showed similar levels of gene excision (data not shown). We chose 200 nM rapamycin for 8 h as the standard treatment condition for subsequent experiments.

Next, we tested the specificity of rapamycin-dependent conditional excision of *gfp* in Δcg6DC4:pHH3GFP parasites along with the parental Δcg6DC4 parasites and WT containing pHH3-SP-loxPint-GFP-loxP plasmid, WT: pHH3GFP. All the parasites were sorbitol synchronized followed by treatment with (+) or without (-) rapamycin. Diagnostic PCR with MSP3F/hrp2R primer set resulted in 1471 bp and 670 bp amplicons in the Δcg6DC4:pHH3GFP parasites under (-) and (+) rapamycin conditions, respectively (Fig. 3a and Fig. S1a). In the WT: pHH3GFP parasites an amplicon of 1471 bp was obtained under both (+) and (-) rapamycin condition (Fig. 3a) suggesting that rapamycin by itself is not responsible for conditional excision of *gfp* sequence. The efficiency of *gfp* excision in the presence of rapamycin is 96 ± 2% ($n = 3$). To rule out non-specific, rapamycin-mediated, excision in Δcg6DC4:pHH3GFP parasites, the *cdpk5* gene was amplified using the primer set CK5FLGSTHISP42_F/ CK5FLGSTHISP42_R. An amplicon of the expected size (1730 bp) was obtained in all the parasites irrespective of the treatment condition (Fig. 3a). No amplification was observed in the parental Δcg6DC4 parasites with the MSP3F/hrp2R primer set (Fig. S1a). Primer set F1/R1, used as loading control, resulted in an amplicon of desired size (1842 bp) in the Δcg6DC4 parasites (Fig. S1a). We also tested the expression of GFP in the parasites by Western blot using anti-GFP Ab. An intense band corresponding to the desired size of GFP was seen in Δcg6DC4:pHH3GFP parasites in the absence of rapamycin while a very faint band was visible in the presence of rapamycin (Fig. 3b and Fig. S1b). As expected, in the WT: pHH3GFP parasites GFP signal was

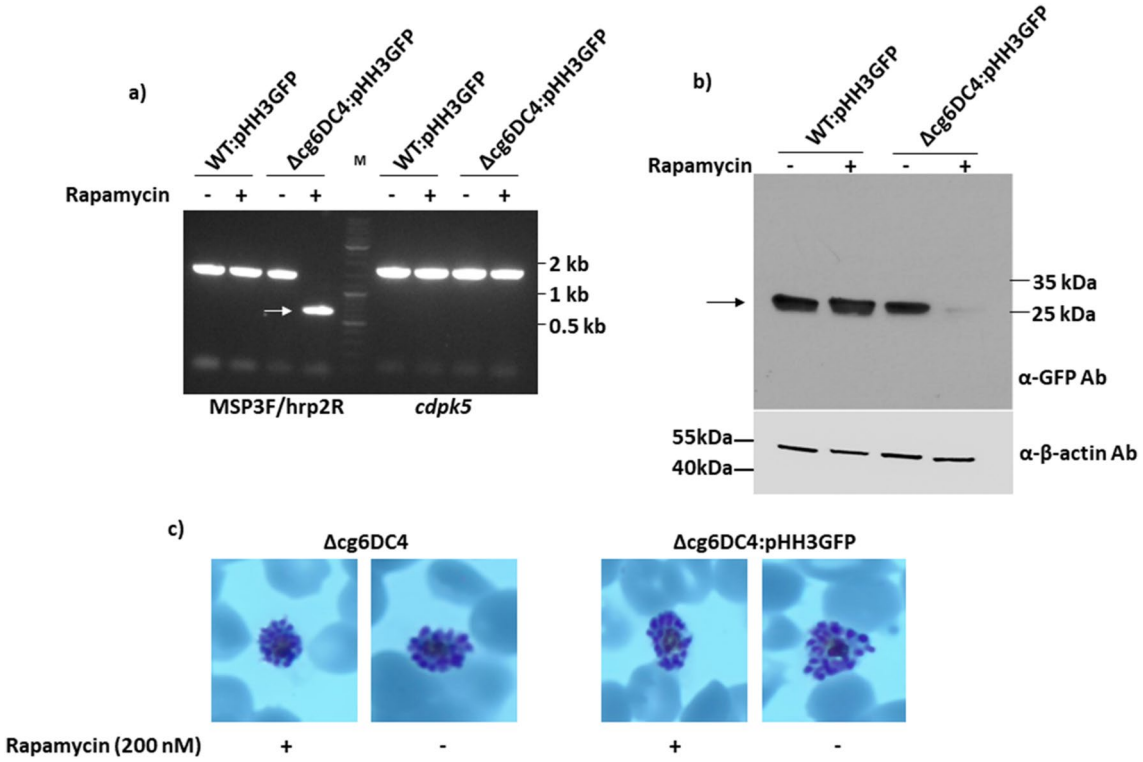

Figure 3. Rapamycin mediated regulation of *gfp*is specific to the Δcg6DC4:pHH3GFP parasites. (**a**) PCR based verification of rapamycin dependent *gfp* excision in Δcg6DC4:pHH3GFP parasites. The pHH3-SP-loxPint-GFP-loxP containing Δcg6DC4 (Δcg6DC4:pHH3GFP) and wild type (WT, WT: pHH3GFP) parasites were treated with 200 nM rapamycin for 8 h. Rapamycin induced excision of *gfp* was verified using primer set, MSP3F/hrp2R. The primer set MSP3F/hrp2R results in amplicons of 1471 bp and 670 bp corresponding to the intact and excised *gfp* sequence, respectively. No excision of *gfp* sequence was seen in the WT: pHH3GFP parasites irrespective of the treatment condition. Amplicon corresponding to the truncated *gfp* sequence is indicated by a white arrow. An amplicon of 1730 bp corresponding to *cdpk5* gene was amplified in absence and presence of rapamycin in both the parasites. M- GeneRuler DNA Ladder Mix. (**b**) Western blot to verify conditional downregulation of GFP expression in Δcg6DC4:pHH3GFP parasites. Lysates from the parasites, Δcg6DC4:pHH3GFP and WT: pHH3GFP, treated with (+) or without (-) rapamycin were separated on SDS-PAGE and probed with anti-GFP antibodies. A band corresponding to the desired size of GFP protein (shown with a black arrow) was visible in WT: pHH3GFP parasites irrespective of rapamycin treatment and Δcg6DC4:pHH3GFP parasites only in absence of rapamycin (black arrow). A faint band corresponding to GFP is visible in the presence of rapamycin in the Δcg6DC4:pHH3GFP parasites. Anti-β-actin antibodies were used as loading control. (**c**) Treatment of Δcg6DC4:pHH3GFP and Δcg6DC4 parasites with 200 nM rapamycin for 8 h did not show any perceptible effect on the morphology and health of the parasites as indicated by the Giemsa smear of mature schizonts.

visible under both (+) and (-) rapamycin conditions corroborating the result obtained in the PCR assay (Fig. 3b). All the samples probed in a parallel Western blot with anti-β-actin Ab showed equal loading (Fig. 3b). No GFP expression was observed in Δcg6DC4 under (+) and (-) rapamycin condition (Fig. S1b). Taken together, these results conclusively demonstrate the ability of Δcg6DC4 parasites to mediate robust, specific, and rapamycin-dependent conditional regulation of exogenously encoded *gfp* sequence floxed with loxP sites. Giemsa-stained late-stage schizonts from Δcg6DC4:pHH3GFP and Δcg6DC4 parasites under (+) and (-) rapamycin conditions were observed under a light microscope to check for any rapamycin-induced abnormalities or defects. The schizonts of both parasites were without any discernible defects under treatment with 200 nM rapamycin for 8 h (Fig. 3c). These results confirm that Δcg6DC4:pHH3GFP parasites exhibit rapamycin-dependent excision of the loxP flanked *gfp* sequence.

DiCre expressing parasites show robust conditional regulation of an endogenous parasite gene

The DiCre expressing Δcg6DC4 parasite shows robust conditional knock-out of the exogenously expressed gene as evident by the downregulation of GFP protein in Δcg6DC4:pHH3GFP in the presence of rapamycin. Next, we wanted to test the utility of Δcg6DC4 in mediating rapamycin-induced excision of an endogenous parasite gene. We chose the functionally uncharacterized gene PF3D7_ 1246000 deemed essential for asexual growth of *P. falciparum*[41]. PF3D7_ 1246000 contains 4 exons and 3 introns and codes for a protein with a predicted

molecular weight of 58 kDa (Fig. 4a; https://Plasmodb.org). No specific domains are found in the protein sequence however, 2 transmembrane domains (7–24 and 444–466 aa) were predicted through InterPro (Fig. 5a; https://www.ebi.ac.uk/interpro/result/InterProScan). To generate a conditional knock-out of PF3D7_ 1246000 we integrated two loxP sites in the target locus using CRISPR-Cas9. The C-terminal region of the first exon was flanked with loxP-int[24] (an artificial intron of *sera2* containing an embedded loxP site) towards the 5' side while another loxP site was integrated in an existing, first intronic sequence (Figs. 4a and 5a). Upon treatment with rapamycin the intervening DNA sequence is deleted and the subsequent reading frame of the truncated gene is altered leading to distorted amino acid sequence and pre-mature termination of protein synthesis (Figs. 4a and 5a). The excised PF3D7_1246000 sequence will encode a truncated protein (1-223 aa) that is devoid of exon 2 and 3 along with the second transmembrane domain (Fig. 5a). A guide sequence specific for the first exon was designed and expressed through pL6-cas9-hDHFR-1246000G plasmid that also expresses Cas9 endonuclease (Fig. 4a). Synthetic gene sequence containing recodonized gene fragment along with the 5' and 3' homology regions was provided in pUC57-1246000loxP plasmid to repair the double strand breaks (Fig. 4a). Two independent clones, C1 and C2, of PF3D7_1246000loxP (Δcg6DC4:6000loxP C1 and Δcg6DC4:6000loxP C2) were verified through a diagnostic PCR for correct integration of loxP sites. Amplicons of desired sizes of 778 bp and 915 bp were obtained with the prime set 5/4 in Δcg6DC4 and the two clones of Δcg6DC4:6000loxP, respectively (Fig. 4b). Primer 4 is located outside the boundary of 3' homology region therefore, the amplicon obtained through primer set 5/4 will not amplify from the rescue plasmid. Primer set 1/2 resulted in an amplicon of the expected size of 452 bp only in Δcg6DC4 since the reverse primer, 2, was specific for the wild-type gene sequence (Fig. 4b). A specific amplicon of 506 bp was obtained in both the clones of Δcg6DC4:6000loxP with the primer set 1/3, since the reverse primer, 3, was specific for the recodonized PF3D7_1246000 gene fragment

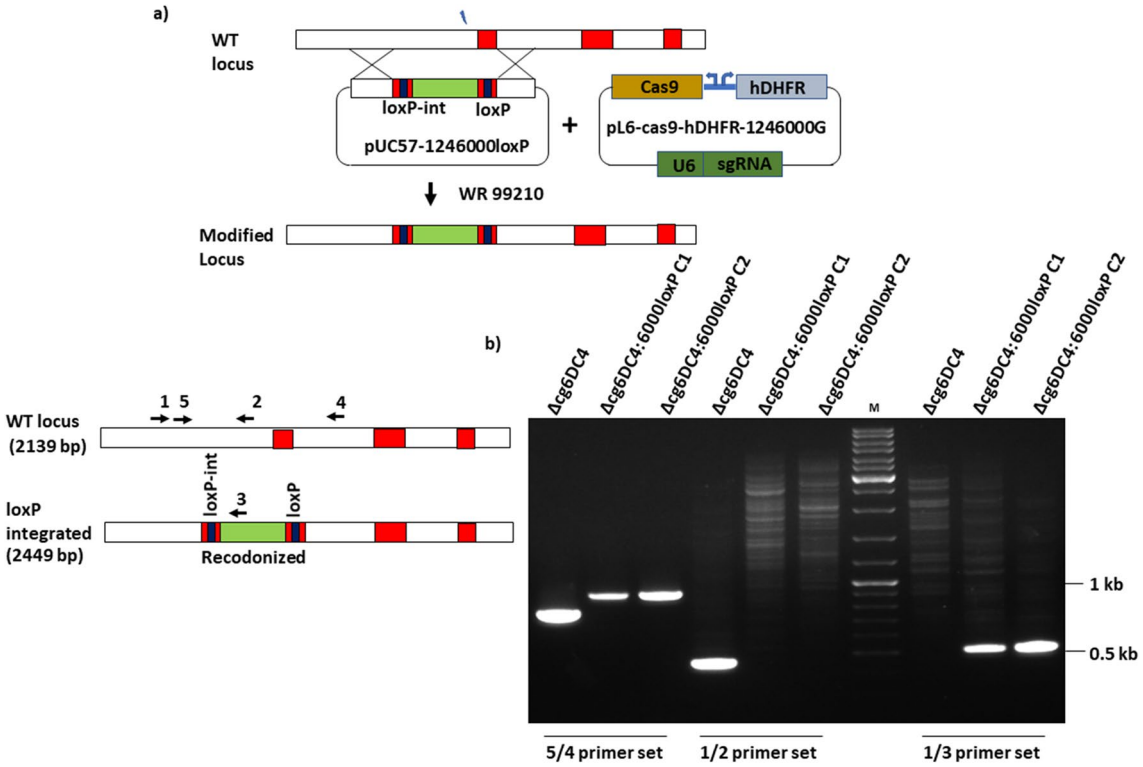

Figure 4. PCR confirmation of loxP integration in the target PF3D7_1246000 locus through CRISPR-Cas9 gene editing. **a**) Schematic diagram representing the strategy used to integrate loxP sites in the PF3D7_1246000 locus. Cas9 endonuclease expressed from pL6-cas9-hDHFR-1246000G was targeted to the 3' end of exon 1. The double strand breaks were repaired by the rescue plasmid containing a synthetic gene sequence comprising of 5' and 3' homology regions corresponding to the target locus. The homology regions flank a recodonized gene fragment containing loxP sites in the intronic sequences. loxP-int represents an artificial intron of *sera2* containing an embedded loxP site; another loxP site was placed in the existing intron 1 sequence. After the desired double homologous recombination, a modified locus containing with two loxP sites flanking the recodonized region is generated. **b**) PCR verification of desired loxP integration in the clonal Δcg6DC4:6000loxP parasites. Primer set 5/4 that specifically amplifies the WT and the modified locus results in amplicons of expected sizes of 778 bp and 915 bp in Δcg6DC4 and both the clones of Δcg6DC4:6000loxP. Primer pair 1/2 specifically amplifies WT locus resulting in an amplicon of 452 bp while primer set 1/3 specifically produces an amplicon of 506 bp in both the clones of Δcg6DC4:6000loxP. Binding positions of all the primers is shown in the scheme on the left for the WT locus and the modified locus after loxP integration. M- GeneRuler DNA Ladder Mix.

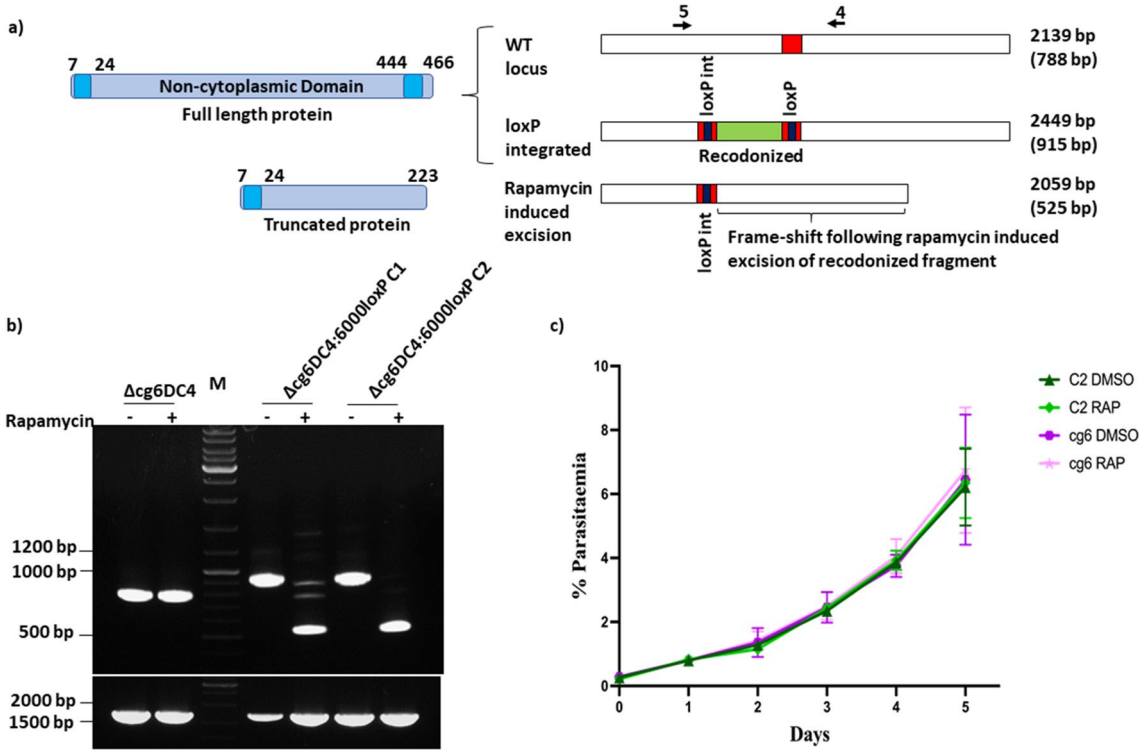

Figure 5. The DiCre expressing Δcg6DC4 parasites show robust, rapamycin dependent conditional excision of PF3D7_1246000 gene. (**a**) Schematic representation of the PF3D7_1246000 gene and corresponding proteins encoded by the WT, loxP integrated and the excised locus. Numbers on the right denote gene size and the amplicons obtained using primer set 5/4 are shown in parenthesis for all the loci. The WT and the loxP modified locus encode full length, the PF3D7_1246000 protein while a truncated protein (1-223 aa) is encoded by the excised locus generated upon rapamycin treatment. (**b**) PCR verification of rapamycin dependent excision of PF3D7_1246000. Samples containing genomic DNA from late stage Δcg6DC4, Δcg6DC4:6000loxP C1, and Δcg6DC4:6000loxP C2 parasites either treated (+) or not treated (-) with rapamycin were used for PCR amplification using primer set 5/4. In Δcg6DC4:6000loxP C1, and Δcg6DC4:6000loxP C2 parasites amplicons of 915 bp and 525 bp were obtained in the absence and presence of rapamycin, respectively. An amplicon of 778 bp was obtained in the Δcg6DC4 parasites irrespective of the treatment condition. The *cdpk5* gene was amplified (1730 bp) in all the parasites in presence and absence of rapamycin (lower inset). M- GeneRuler DNA Ladder Mix. (**c**) The Δcg6DC4:6000loxP clonal parasite show similar growth profile as the parental Δcg6DC4 parasite in presence and absence of rapamycin. Highly synchronized Δcg6DC4 (cg6) and Δcg6DC4:6000loxP C2 (C2) parasites were equally divided into two separate flasks and treated either with (RAP) or without (DMSO) rapamycin. Small aliquots were drawn on each day, treated with SYBR Green I and the parasitemia was estimated using flow cytometry. The % (percent) parasitemia on the Y-axis was plotted against number of days on the X-axis. Total 100,000 cells were acquired in singlets from 3 biological experiments for the estimation of parasitemia under each condition. The graph is plotted using GraphPad Prism 9. The difference in the parasitemia under DMSO and RAP condition for cg6 and C2 was calculated using multiple paired t-tests ($p > 0.05$; not significant). Error bars represent Mean ± SEM.

(Fig. 4b). We further confirmed the integration of the loxP sites at the desired location in PF3D7_1246000 through DNA sequencing. A gene segment comprising the modified region was PCR amplified from Clone 2 of Δcg6:6000loxP using the primer set 5/4 and sequenced in the forward and reverse directions. The DNA sequence of the PCR amplified product perfectly aligned with the desired sequence (Supplementary file). These results confirm the successful integration of loxP sites in the PF3D7_1246000 locus with the subsequent modification of the intervening DNA sequence.

Next, we tested the conditional regulation of PF3D7_1246000 in Δcg6DC4:6000loxP parasite clones. To this end, the clonal parasites of Δcg6DC4:6000loxP were treated with rapamycin or DMSO along with the parental Δcg6DC4 parasites and analyzed in the same cycle (Fig. 5b and Fig. S2) and subsequent cycle (Fig. S2) through PCR. Samples containing template DNA were prepared from the DMSO and RAP-treated parasites and amplified using primer set 5/4. An amplicon of 915 bp was obtained in both the clones of Δcg6DC4:6000loxP in DMSO condition while a smaller, truncated amplicon of the expected size of 525 bp was obtained in the presence of RAP (Fig. 5b and Fig. S2). An amplicon of 778 bp was obtained in the parental Δcg6DC4 parasites in both DMSO and RAP conditions (Fig. 5b and Fig. S2). To test if the rapamycin-mediated gene excision is specific for the PF3D7_1246000 locus, we amplified the full-length *cdpk5* gene sequence with the primer set CK5FLGSTHISP42_F/ CK5FLGSTHISP42_R. As expected, an amplicon of 1730 bp was obtained in all the

parasites irrespective of the treatment condition (Fig. 5b). In clone 1 of Δcg6DC4:6000loxP parasites, we noticed the presence of some unexcised gene that was very faint in the C2 clone (Fig. 5b and Fig. S2). To further verify the boundary and the desired excision of PF3D7_1246000 in the presence of rapamycin, we sequenced the 525 bp PCR product obtained using 5/4 primer set from Clone 2 of Δcg6:6000loxP in RAP condition in the forward and reverse directions (Supplementary file). The DNA sequence of the PCR product perfectly aligned with the desired sequence (Supplementary file). A stop codon at the expected position was also present in the sequenced fragment (highlighted red; supplementary file). These results confirm that the conditional knock-out of PF3D7_1246000 is mediated by rapamycin only in the Δcg6DC4 parasite background and is specific to the modified target locus.

PF3D7_1246000 is not essential for the asexual replication of the parasite

Next, we wanted to test the function of PF3D7_1246000 in the asexual progression of the parasite inside RBCs. To this end, the clone 2 of Δcg6DC4:6000loxP along with the parental Δcg6DC4 parasites were tightly synchronized using percoll-sorbitol. The RAP or DMSO-treated parasites at the trophozoite stage were seeded at 0.2% parasitemia and allowed to progress for 5 days. Daily parasitemia was monitored by FACS, and SYBR Green I assay, and the data was plotted for all the days. The parasitemia of Δcg6DC4:6000loxP was comparable in DMSO and RAP conditions on all the days and was similar to that of the parental Δcg6DC4 parasites (Fig. 5c, and Fig. S3) suggesting that the deletion of PF3D7_1246000 did not affect the parasite growth under in vitro conditions. These results also suggest that the modification of PF3D7_1246000 locus through the integration of loxP sites did not affect the parasite fitness. Moreover, there were no perceptible defects in the morphology and health of the parasite in the RAP condition compared to that of the DMSO control as evident through the Giemsa smears (Fig. S4). These results suggest that ablation of PF3D7_1246000 is not deleterious for the parasite growth and as such the gene may not be essential for the asexual replication of the parasite within red blood cells under in vitro culture conditions.

Discussion

Dimerizable Cre recombinase is a powerful tool to investigate the function of essential genes. Initially, the lack of effective conditional systems to regulate the activity of Cre recombinase impeded the adoption of the Cre-lox technique in malaria parasite[42,43]. The problem was overcome by the development of a modified Cre enzyme called Dimerizable Cre recombinase that could be activated conditionally[19,20]. In this technique, two inactive fragments of Cre recombinase, Cre59 and Cre60 are reconstituted into a functionally active enzyme either in the presence of a small molecule or light[21–23]. DiCre cassette has been inserted at different loci in malaria parasites to generate parasite strains that constitutively express inactive DiCre fragments[25–27,32]. These DiCre-expressing strains have been used to understand the function of various genes in blood stage development of malaria parasite[26,32,44–51]. To further broaden the repertoire and availability of stable DiCre-expressing parasite strains, herein we have inserted a DiCre expression cassette in the *cg6* locus of the NF54 wild-type parasite. The *cg6* locus is redundant for the asexual replication of malaria parasite[31,34,52,53]. In corroboration with the previous reports, the insertion of DiCre into the *cg6* locus had no negative effect on the parasite growth in our study as well. In a recent study, disrupted *cg6* locus in NF54^{attB} parasite[31] was used to integrate a plasmid containing DiCre and TetR-DOZI to study metabolic changes in parasites lacking essential TCA cycle enzymes[25]. The entire plasmid was integrated into the *cg6* locus through *attB*/*attP* recombination resulting in the existence of a BSD resistance cassette in the final DiCre expressing parasite strain[25]. Marker-free gene editing through CRISPR-Cas9 is used to generate Δcg6DC4 parasites in this study allowing usage of any of the limited number of positive selection markers available for genetic studies in malaria parasite[54]. We used two different concentrations of rapamycin, 100 nM and 200 nM, to test the excision of genes with adjacent loxP sites. There were no growth defects in the asexual replication of the parasite at both the concentrations of rapamycin tested. Previous studies have also employed similar concentrations of rapamycin for conditional knock-out of *ama1*[26] and *sera5* 3'UTR region[27] without any inhibitory effect on the parasite growth. Rapamycin is toxic to the parasite at a much higher concentration[55] than the one employed in this and earlier studies[26,27]. The DiCre-expressing Δcg6DC4 parasites generated in this study show robust and highly efficient excision of gene fragments flanked with loxP sites. Importantly, there was a marked reduction in GFP expression in the presence of rapamycin. Moreover, there was no excision of the loxP-tagged *gfp* sequence under DMSO treatment which showed the high specificity of the DiCre system. In a previous study conditional deletion of *ama1* through episomal expression of DiCre resulted in ~80% reduction in AMA1 protein expression[26]. Other studies that employed DiCre expression through episomal plasmid could achieve 50–60% depletion of GoI in the presence of rapamycin in the first treatment cycle[56]. The incomplete depletion of AMA1 and other target proteins might be due to the usage of a weaker ef1α promoter that drove the expression of a DiCre fragment[26,56]. As opposed to a weaker promoter, the expression of DiCre fragments in Δcg6DC4 parasites is driven by strong and constitutively active *BiP* and *hsp86* promoters[27,35]. DiCre parasite lines generated by other groups via disruption of different loci showed robust and >98% excision rates of floxed genes[27,35,57]. With the Δcg6DC4 parasite strain, we achieved 96% excision of loxP floxed *gfp*. Collins et al. reported the generation of the first DiCre parasite line wherein the DiCre cassette was integrated within 3' UTR of *sera5* locus through single recombination[27]. Spontaneous excision of the DiCre cassette due to homologous recombination caused reversion to WT in these parasites[27]. Later the duplicated 5' *sera5* sequence, downstream to the DiCre cassette was removed using CRISPR-Cas9 to prevent reversion by homologous recombination[58]. In addition, the DiCre cassette was integrated in the *p230p* and *pfs47* which are not required for the asexual replication and sexual development of the parasite[33,35]. These parasite lines have been widely used for functional characterization of malaria parasite genes[45,58–62].

Since deletion of *cg6* does not hamper the completion of the malaria parasite life-cycle[31,34,53], therefore, the Δcg6DC4 parasites may be used for functional analysis of genes in other stages of parasite development

within mosquito and hepatocytes. Inducible gametocyte-producing parasite lines were generated by disrupting the *cg6* locus for conditional overexpression of gametocyte development 1, a critical factor required for sexual commitment[53,63]. The Δcg6DC4 parasites may also be utilized to study genes having enzymatic and scaffolding roles by integrating multiple incompatible loxP sites in artificial or existing intronic sequences[64]. Moreover, the *cg6* locus may be targeted in other *Plasmodium* species of medical importance to generate transgenic parasites that can support conditional knock-out of target genes in the presence of rapamycin.

PF3D7_1246000 encodes a functionally uncharacterized protein that was found to be essential for *P. falciparum* asexual growth[41]. However, its ortholog in *P. berghei* (PBANKA_145910) has been successfully disrupted in a whole genome knock-out screen[65]. Our results suggest that PF3D7_1246000 may not be required for normal asexual replication of parasites as conditional deletion of the gene doesn't seem to have any apparent deleterious effects on the health and morphology of the parasites under the in vitro conditions employed. Our conditional knock-out strategy results in the expression of a remnant portion of PF3D7_1246000 devoid of the C-terminal transmembrane domain along with amino acid sequence following Lys 223 that encompasses exon 2 and 3. In the absence of exons 2, and 3 and the membrane anchoring transmembrane domain, it is highly unlikely that the truncated protein will be correctly localized within the parasite and execute its native function. However, there may be a possibility that the native protein interacts with another membrane resident protein via its N-terminus and still exerts some function even when truncated. Therefore, the possibility that the remnant, C-terminal-deleted protein can carry out the functional role following rapamycin treatment cannot be completely ruled out. It is thus not excluded that the truncated protein still provides enough function to maintain parasite growth. Available transcriptomics data suggest high expression of PF3D7_1246000 in sexual stages compared to the asexual stages [https://Plasmodb.org]. It will be interesting to test the function of PF3D7_1246000 in the sexual development of the parasite. Complete knock-out of the full CDS either through permanent replacement with a resistance marker or conditional disruption will be useful to unambiguously show the non-essentiality of PF3D7_1246000 in the asexual proliferation of the parasite within RBCs. Nevertheless, in this study, we show the successful integration of DiCre cassette in *cg6* locus of WT parasite and also the utility of Δcg6DC4 in mediating rapid, conditional, and site-specific excision of genes floxed with loxP sites in a single cycle of asexual replication. The Δcg6DC4 parasites generated in this study are an added resource that can be used for the functional characterization of essential parasite genes for future drug and vaccine development against malaria.

Methods

Malaria parasite *in vitro* culture and maintenance

Plasmodium falciparum NF54[37] was maintained at 2% hematocrit in O + human red blood cells obtained from Rotary Blood Centre, New Delhi, India. The culture medium composition used for growing the parasites is RPMI 1640 with L-glutamine (ThermoFisher Scientific), 0.25% Albumax II Lipid-Rich BSA (ThermoFisher Scientific), 5% heat-inactivated AB + human sera, 25 mM HEPES (Merck Millipore), 50 µg/mL Hypoxanthine (Merck Millipore), 10 µg/mL gentamicin (ThermoFisher Scientific), 25 mM Sodium bicarbonate (Sigma-Aldrich). The parasites were maintained at 37°C under the environment of 5% O_2 and 5% CO_2 balanced with N_2 as described earlier[66].

Construction of plasmids and generation of gene edited parasites

Parasites with DiCre integrated in cg6 locus

The DiCre containing plasmid, pBSP230pDiCre (kind gift from Ellen Knuepfer and Anthony Holder) was modified to integrate the DiCre cassette in the *cg6* locus. For this, the DiCre cassette was flanked with 5' and 3' homology regions corresponding to the DNA sequence of *cg6*. The 3' HR (330 bp) was amplified using the primer pair Alt3HRCG6F and Alt3HRCG6R for cloning between SpeI and KpnI. The 5' HR (368 bp) was amplified using the primer pair Alt5HRCG6F and Alt5HRCG6R for cloning between SacI and AflII. Cloning of both the 5' and 3' HR in the final plasmid, pBS-cg6-DiCre was verified by a diagnostic PCR and DNA sequencing. The pL6-eGFP plasmid[67] was modified by substituting the negative selection marker, FCU with Cas9 endonuclease DNA sequence. The Cas9 DNA sequence along with 5' and 3' utr regions was excised from pUF1-Cas9 plasmid[67] using NotI and SacII and cloned in the compatible restriction sites in the pL6eGFP plasmid. Additionally, the positive selection marker, hDHFR, in pL6eGFP was substituted with DHODH from pUF1Cas9 using AflII and EcoRI. The final plasmid containing Cas9 along with DHODH was named pL6-Cas9-DHODH. The pL6-Cas9-DHODH was used for cloning a 20-nucleotide guide region (5' CCTTTTGTTACCCTTTGTT G 3'), corresponding to the *cg6* DNA sequence, using the oligo pair Gcg6F and Gcg6R. Protocol as reported earlier was used for cloning the guide sequence in pL6-Cas9-DHODH[68]. Briefly, the oligos were annealed in the annealing buffer by first heating for 5 min at 95 °C followed by slow cooling to ambient temperature. Diluted annealed oligos were cloned in the target pL6-Cas9-DHODH plasmid using In-Fusion (Clontech, Takara) following the manufacturer's instructions. The DNA sequence of the guide region along with the tracrRNA was verified by Sanger DNA sequencing and the plasmid was renamed as pL6-Cas9-DHODH-cg6G. The two plasmids, pBS-cg6-DiCre, and pL6-Cas9-DHODH-cg6G, 50 µg each, were co-transfected in the ring stage parasite using electroporation with the following parameters: 310 volts, 950 µF and ∞ resistance as described earlier[36,37]. The transfected parasites were switched to 150 nM DSM267[38] containing medium after 24–48 h depending on the parasitemia of the transfected parasites. Drug-resistant parasites were obtained around 2–3 weeks post-transfection. The transfected parasites were cloned using the limiting dilution method. The clonal parasites were tested for the desired integration of DiCre cassette at *cg6* locus using diagnostic PCR with three different primer sets: F1/R1, F1/R2, and F2/R3 (see Table S1 in Supplementary File for primer sequence). The *cg6* disrupted parasites with DiCre integration are named as $\Delta cg6^{Dicre}$ and the two clonal parasites obtained are likewise named $\Delta cg6^{DicreC4}$ and $\Delta cg6^{DicreC8}$. The $\Delta cg6^{DicreC4}$ parasites were used for all subsequent experiments and are designated Δcg6DC4.

Parasites with loxP sites integrated in PF3D7_1246000 locus

To generate a conditional knock-out parasite for PF3D7_1246000, two loxP sites were integrated into the target locus CDS for conditional regulation of its expression by rapamycin. To achieve this, we designed a synthetic construct wherein a recodonized gene fragment (RCGF; 224–312 aa) was flanked by loxP sites. A loxP site towards an upstream portion was embedded in an artificial intron while an existing intron towards the downstream portion was used to integrate another loxP site. Two homology regions of 207 bp and 287 bp corresponding to the immediate 5' and 3' DNA sequence of PF3D7_1246000 were selected for homology-directed repair of the targeted PF3D7_1246000 locus. The complete gene sequence in the pUC57 plasmid (GenScript) was named pUC57-1246000loxP and used as a rescue plasmid for homology-directed repair through CRISPR-mediated gene editing. For introducing a double-strand break in the target locus, we used pL6-Cas9-hDHFR plasmid to clone a 20-nucleotide guide DNA sequence (5' CACTCTGCAAAACATCAGAC 3'), immediately upstream of a PAM sequence (5' GGG 3'), corresponding to the 1,246,000 CDS. The guide region was cloned with an oligo pair: 1246000GF and 1246000GR using In-fusion as described above. The DNA sequence of the guide along with the tracrRNA in the sgRNA was verified by Sanger DNA sequencing. The plasmid containing the guide sequence was named pL6-cas9-hDHFR-1246000G. The two plasmids, pUC57-1246000loxP and pL6-cas9-hDHFR-1246000G were co-transfected in Δcg6DC4 parasites as described above. Since the pL6-cas9-hDHFR-1246000G plasmid contains hDHFR as the positive selection marker for loxP integration, therefore, 2.0 nM WR99210 was used instead of DSM267 for the positive selection of transfected parasites. After two weeks of positive selection, drug-resistant parasites were obtained. WR99210-resistant parasites were cloned using limiting dilution. Four independent clones of Δcg6DC4: 6000loxP (A5, C1, C2, and D1) were obtained out of which two clones (C1 and C2) were verified for the desired integration of loxP sites through a diagnostic PCR with two different sets of primer pair: 5/4 and 1/3. To check the contamination of the parental parasite in Δcg6DC4:6000loxP clones, a primer set (1/2) was used. The primer set, 1/2 was specifically designed to amplify only the parental PF3D7_1246000 gene sequence.

Generation of GFP expressing parasites

To generate parasites that exogenously express a green fluorescent protein (GFP), the ring stage Δcg6DC4 parasites were transfected with pHH3-SP-loxPint-GFP-loxP (a kind gift from Ellen Knuepfer and Anthony Holder) with the parameters as defined in the above section. As a control for rapamycin-mediated excision, the WT parasites were also transfected with the pHH3-SP-loxPint-GFP-loxP plasmid. The transfected parasites were selected with 2 µg/mL of blasticidin S (ThermoFisher Scientific) since the plasmid pHH3-SP-loxPint-GFP-loxP contains blasticidin S deaminase positive selection marker. After two weeks of positive selection, drug-resistant parasites were obtained that were verified for the presence of the plasmid using diagnostic PCR. The pHH3-SP-loxPint-GFP-loxP containing Δcg6DC4 and WT parasites were named Δcg6DC4:pHH3GFP and WT: pHH3GFP, respectively. Primer pair MSP3F/hrp2R was used to amplify the entire *gfp* sequence including the loxP sites using Primestar Max DNA polymerase using standard PCR amplification conditions. The control primer set, F1/R1 specific for the disrupted *cg6* locus was used as a control in the Δcg6DC4:pHH3GFP and Δcg6DC4 parasites.

To test rapamycin-mediated excision of *gfp* DNA sequence in Δcg6DC4:pHH3GFP, the parasites were sorbitol synchronized, divided equally into two separate flasks, and treated with different concentrations of rapamycin (100 nM and 200 nM) for 4 and 8 h. The WT: pHH3GFP and Δcg6DC4 parasites treated in the same manner were used as controls. The parasites were washed 2x with incomplete RPMI1640 and allowed to progress till the late schizont stage. Mature schizonts were harvested by centrifugation followed by saponin lysis. The parasites were extensively washed with 1X PBS and samples were prepared for PCR and Western blot analysis. For PCR analysis, the parasite pellet was resuspended in RNase-DNase free water and heated at 95 °C for 10 min followed by centrifugation at 14,000 g for 10 min at 4 °C. The supernatant was collected in a separate tube for PCR analysis. To determine the percentage excision, the band intensity of the *gfp* amplicon (1471 bp) obtained with the primer set MSP3F/hrp2R under DMSO and RAP conditions was calculated using Image J software [www.imagej.net]. The intensities of the bands were normalized with the control amplicon obtained using F1/R1 primer set. Percentage excision of *gfp* was calculated using the formula: 100-[100*(normalized intensity of *gfp* amplicon (1471 bp) in presence of rapamycin/normalized intensity of *gfp* amplicon (1471 bp) in absence of rapamycin)]. The data was obtained from 3 independent experiments and standard deviation was calculated using GraphPad Prism 9. Western blot analysis for GFP protein detection is described below.

Evaluation of the excision of PF3D7_1246000 in presence of rapamycin

To evaluate the effect of rapamycin treatment on Δcg6DC4:6000loxP parasites containing floxed PF3D7_1246000, the parasites were synchronized using the percoll-sorbitol method. Briefly, the parasites enriched by the percoll-sorbitol method were allowed to invade fresh RBCs for 4 h. After 4 h of invasion, the parasites were treated with sorbitol to eliminate the remaining mature parasites and obtain highly synchronized parasites of 0–4 HPI (hour post-invasion). The sorbitol synchronized parasites were equally divided into two separate flasks and treated with 200 nM rapamycin for 8 h followed by two washes of incomplete RPMI1640 to completely remove rapamycin and DMSO. The treated parasites were allowed to progress to the late schizont stage in the same cycle or the next cycle. The parasites were harvested by centrifugation followed by saponin treatment. Template DNA was prepared from the rapamycin and DMSO-treated parasites and amplified with the primer set 5/4 using PrimeSTAR Max DNA polymerase (Clontech) employing the amplification parameters prescribed by the manufacturer or used in previous studies[37,68]. The full-length cdpk5 gene was amplified using the primer set CK5FLGSTHISP42_F/ CK5FLGSTHISP42_R.

Western blot for detection of GFP in transgenic parasites

Western blot analysis was used to detect GFP protein in Δcg6DC4:pHH3GFP and WT: pHH3GFP parasites. The parasite pellets generated in 'Generation of GFP expressing parasites' were resuspended in a modified 1X RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 50 mM Tris (pH 8.0), 1 mM PMSF and 1x protease inhibitor cocktail (Roche Life Science) for 1 h in ice followed by centrifugation at 14,000 g for 15 min at 4°C. The supernatant was mixed with SDS loading dye and incubated at 95°C for 10 min. The sample was centrifuged at 14,000 g for 10 min at 4°C. The supernatant was collected in a separate centrifuge tube and separated on a 10–12% SDS-PAGE gel followed by transfer onto a PVDF membrane. The PVDF membrane containing the transferred parasite proteins was blocked with 5% skimmed milk in 1X Tris-buffered Saline (TBS) buffer containing 0.1% Tween-20 for 1 h at RT. The PVDF membrane was then incubated with 1:1000 dilution of anti-GFP monoclonal antibody (GF28R, ThermoFisher Scientific) or 1:5000 dilution of anti-β-actin antibody (A2228; Sigma-Aldrich) in blocking buffer for 1 h at RT followed by 3X washes of 1X TBST. The blot was then incubated with goat anti-rabbit or goat anti-mouse IgG peroxidase-conjugated secondary antibody in blocking buffer for 1 h at RT followed by 3X washes of 1X TBST. The blot was finally developed with a chemiluminescent substrate and exposed on either X-ray film or scanned through Biorad ChemiDoc XRS+.

Asexual growth assay using SYBR Green I assay

Highly synchronized (0–4 HPI) Δcg6DC4:6000loxP C2 parasites, as described above, were distributed equally into two separate flasks and treated either with (+; RAP) or without (-; DMSO) rapamycin (200 nM) for 8 h. The parasites were washed 2 times with incomplete RPMI1640 and allowed to progress to the trophozoite stage. The parasitemia of the parasites was set at 0.2% and allowed to grow under optimum conditions as described under 'Malaria parasite in vitro culture and maintenance'. Aliquots were drawn every 24 h for 5 days and stored in -80 degrees for 5 days. The parasites were processed as described earlier[39,69,70]. Briefly, the parasites were lyzed in a lysis buffer of the following composition: Tris (20 mM; pH 7.5), EDTA (5 mM), and saponin (0.008%; wt/vol). The plate was incubated for 30 min at RT. The fluorescence readings were acquired at the excitation and emission wavelengths of 485 nm and 520 nm, respectively in Fluostar Optima (BMG Labtech) plate reader with a gain of 1000. Two experiments were performed in triplicate. The graphs were plotted with the number of days on the X-axis against fluorescence on the Y-axis using GraphPad Prism 9. Statistical significance was calculated using a t-test.

For comparing the growth profile of $\Delta cg6^{DicreC4}$ and $\Delta cg6^{DicreC8}$ with the WT parasites, synchronization was done with two consecutive cycles of sorbitol[71] followed by seeding of the parasites at 0.2% parasitemia in a 24 well plate in triplicate. The samples were processed for SYBR Green I as described above. One experiment in triplicate was performed. The graphs were plotted with the number of days on the X-axis against fluorescence on the Y-axis using GraphPad Prism 9. Statistical significance was calculated using a t-test.

Asexual growth assay using FACS

Highly synchronized (0–4 HPI) Δcg6DC4:6000loxP C2 and Δcg6DC4 parasites, as described above, were equally divided into two separate flasks and were treated either with (+; RAP) or without (-; DMSO) rapamycin (200 nM) for 8 h. The parasites were washed 2 times with incomplete RPMI1640 and allowed to progress to the trophozoite stage. The parasitemia of all the parasites was set at 0.2% and allowed to grow under optimum conditions as described under 'Malaria parasite in vitro culture and maintenance'. Aliquots were drawn every 24 h for 5 days and stained with SYBR Green I (ThermoFisher Scientific) nucleic acid stain as described previously[39,40]. Briefly, 20 µl of suspended parasite culture at 2% hematocrit was washed twice with 1X PBS and resuspended in the final volume of 380 µl of 1X PBS. 20 µl of SYBR Green I (1:100 dilution) was added to each vial and incubated for 25 min at RT. The parasites were then centrifuged at 500 g for 5 min and washed twice with 1X PBS. Finally, the parasites were resuspended in 500 µl of 1X PBS for the acquisition of SYBR Green I stained cells in BD FACSCalibur Flow Cytometer. A total of 100,000 events per sample were acquired and initial gating was done with stained, uninfected RBCs to assess RBCs autofluorescence using BD CellQuest Pro software version 5.1.1. The % (percent) parasitemia under each condition was plotted on the Y-axis against 'Days' on the X-axis using GraphPad Prism 9. Three biological experiments were performed in singlet to compare the growth profiles of Δcg6DC4:6000loxP C2 and Δcg6DC4 parasites under DMSO and rapamycin conditions through FACS. The statistical significance between the two parasites under two different conditions was calculated through GraphPad Prism 9 using a paired t-test.

Light microscopy

Microscopic images of Δcg6DC4:6000loxP C2 parasites under (+) and (-) rapamycin conditions were acquired for the early ring (ER), late ring (LR), trophozoite (T), early schizont (ES), and late schizont (LS) through 100X oil immersion objective in Zeiss Primostar Trinocular Microscope with axiocam 208 color camera.

Data availability

All data generated or analysed in this study are included in this manuscript and its Supplementary Information file. The raw data is also available for analysis and reference, if required. Request may be sent to bansal.abhisheka@gmail.com. Full Western Blots and Agarose Gels are provided in Supplementary Figures S5-S12.

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Author contributions

AB wrote the manuscript; AB generated the transgenic parasites; AB, MS, HC designed and performed the experiments; AB, MS, HC analysed the data and prepared the figures; All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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