Chapter 5

Protein acyltransferases in *Plasmodium falciparum*

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The results described in the previous chapter (Chapter 4) provided a first look at the DHHC protein family in *Plasmodium* parasites. However, in order to allow for further downstream follow-up studies (for example, comparative site-ID palmitome purification of DHHC knock-out lines) the focus was now shifted to the DHHC protein family in *P. falciparum.*

As described in Section 4.1, there are 12 proteins containing the conserved DHHC domain in *P. falciparum*, all except one (PFB0140w/PfDHHC12) possessing a homologue in *P. berghei*. These DHHC proteins have subsequently been named PfDHHC1 to 12, corresponding to their homologues in *P. berghei* and to their closest homologues in *T. gondii* (Table 4.1). BLAST search also revealed that *P. falciparum* appears to have only one open reading frame annotated to be a putative MBOAT family member [1], the second family of proteins thought to act as PATs. Due to the technical difficulties of genetic manipulation in *P. falciparum*, 5 out of the 12 PfDHHC proteins which appear to be more highly expressed in schizonts (PfDHHC3, 5, 7, 8 and 9) [2], were chosen to be C-terminally tagged with a 3-HA epitope tag, in order to determine their subcellular localisation. Additionally, the MBOAT protein in *P. falciparum* was also chosen to be C-terminally tagged with the 3-HA epitope tag. Gene disruption strategies were also performed on the same five PfDHHC proteins and one PfMBOAT protein in an attempt to investigate the essentiality and function of these PATs in *P. falciparum.*

5.1. Expression and localisation of DHHC and MBOAT proteins in *Plasmodium falciparum*

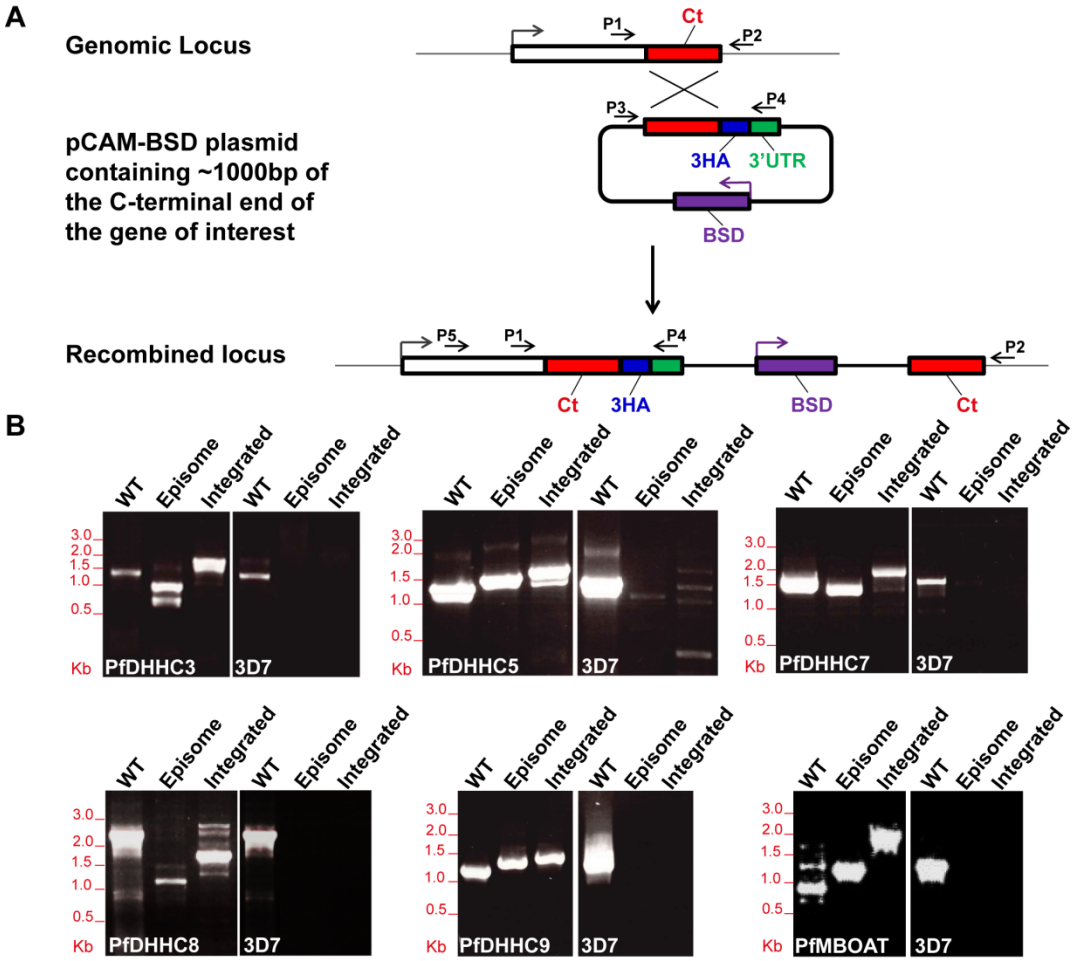
5.1.1. Generation of PfDHHC and PfMBOAT triple-HA (3-HA)-tagged transgenic parasite lines

In order to insert the 3-HA epitope tag into the C-terminus of the PAT genes of interest (PfDHHC3, 5, 7, 8 and 9, and PfMBOAT) at the endogenous loci, approximately 1000 bp of the C-terminal end of each gene was PCR amplified and introduced into a *P. falciparum* tagging vector (pCAM-BSD-3HA) containing the 3-HA epitope tag and the Blasticidin S deaminase gene (*BSD*), which confers resistance to the drug Blasticidin S [3], as described in the Materials and Methods. These PfDHHC and PfMBOAT tagging vectors were then introduced into *P. falciparum* strain 3D7 using the transfection methods described in Materials and Methods, and parasites that had taken up the tagging vector were selected for using resistance to Blasticidin S.

After transfection and drug selection, transgenic parasites resistant to Blasticidin S were obtained for all six PAT proteins studied here. The insertion of the 3-HA epitope tag into the genome was expected to occur via single-crossover recombination at the homologous C-terminal region, as

described in Figure 5.1A, meaning that the tagged DHHC gene would be under the control of its native promoter, which is critical as it removes the possibility of expression-related mis-localisation artefacts which can occur when an episomal strategy is used.

The genomic DNA of all the transgenic parasites were tested for the presence of sequences specific to the integrated construct, the wild-type locus and the episome, by PCR amplification using the primer pairs shown in the schematic in Figure 5.1A, and listed in the table in Figure 5.1B and in Table 2.5. For each of the genes of interest, successful PCR amplification of sequences specific to the integration of the tagging construct into the genomic DNA (primer pair: P5+P4 or P1+P4) indicated that the 3-HA tag was successfully inserted into the C-terminal end of the genes of interest (Figure 5.1B). However, in each of the tagged PfDHHC lines, the continued PCR amplification of sequences specific to the wild-type locus (primer pair: P1+P2) and to the episome (primer pair: P3+P4) indicated that for each transgenic parasite line, a proportion of the parasite population appeared to retain the DHHC tagging vector as an episome, and did not integrate the tagging vectors into the genomic DNA. Nevertheless, transgenic parasites containing the integrated construct could be identified using the expression of the 3-HA epitope tag, as expression of the 3-HA tag was not expected to be detectable when the vector was retained as an episome. For the PfMBOAT transgenic line, sequence specific to the wild-type locus was not amplified at the correct size (Figure 5.1B). This could mean that the wild-type locus is no longer present due to integration of the construct and the bands observed were just background amplification. However, the clear amplification of the sequence specific to integration of the construct indicated that there was indeed integration, and parasites with the integrated construct could be identified by the expression of the 3-HA tag.



Triple-HA-tagged line	Sequence amplified	Primer pair	Expected Size (bp)
PfDHHC3	Wild-type	P1+P2	1406
	Episome	P3+P4	1063
	Integrated	P5+P4	1969
PfDHHC5	Wild-type	P1+P2	1335
	Episome	P3+P4	1536
	Integrated	P5+P4	1699
PfDHHC7	Wild-type	P1+P2	1618
	Episome	P3+P4	1386
	Integrated	P5+P4	2059
PfDHHC8	Wild-type	P1+P2	2160
	Episome	P3+P4	1420
	Integrated	P1+P4	1865
PfDHHC9	Wild-type	P1+P2	1193
	Episome	P3+P4	1347
	Integrated	P5+P4	1474
PfMBOAT	Wild-type	P1+P2	1220
	Episome	P3+P4	1100
	Integrated	P5+P4	2189

Figure 5.1: Generation of PfDHHC and PfMBOAT triple-HA-tagged transgenic lines. (A) Scheme of the strategy used to C-terminally-tag the endogenous locus of the PfDHHC and PfMBOAT genes of interest. The primer positions illustrated here indicate the primers used for genotyping of the 3-HA-tagged transgenic lines. Flags indicate promoter regions. **(B)** Genotyping of the PfDHHC and PfMBOAT tagged transgenic lines by genomic PCR analysis. Primer pairs used for the amplification of sequences specific to the wild-type locus, episome and integrated construct are as listed in the table, along with the expected sizes of the fragments. For all six

transgenic lines, sequences specific to the integrated construct is amplified. For PfDHHC3, 5, 7, 8 and 9, sequences specific to the wild-type locus and the episome are still amplified. For PfMBOAT, sequence specific to the wild-type locus is not amplified at the right size, which may indicate that the wild-type locus is no longer present and the bands observed may be due to background amplification.

5.1.2. Expression of DHHC and MBOAT proteins in P. falciparum

In order to establish whether these tagged proteins were expressed in schizonts as expected, immunoblots of saponin-lysed schizont pellets, collected from all tagged transgenic *P. falciparum* lines, were performed using antibodies against the 3-HA epitope tag. As in *P. berghei*, the expected sizes of the proteins listed on *Plasmo*DB (http://plasmodb.org) ranged from 35 kDa to 160 kDa, depending on the presence or absence of N-terminal ankyrin repeats and the length of the C-terminal region. Immunoblot of the schizont pellets resulted in single bands which ran at the expected sizes for three of the PfDHHC proteins (PfDHHC5, 7 and 9), as well as for PfMBOAT protein, as shown in Figure 5.2. This indicated that PfDHHC 5, 7, and 9, as well as PfMBOAT, were expressed in *P. falciparum* schizonts. Bands were not detected for PfDHHC3 and 8. This could perhaps indicate that these DHHC proteins are expressed at levels that are too low to be detected by immunoblot or by tagging at the endogenous locus. Interestingly, both homologues of these proteins in *P. berghei* (PbDHHC3 and 8, Section 4.2.2), were successfully detected by immunoblotting. This could imply that the expression levels of these proteins differ between the species, perhaps due to the fact that there is one less DHHC protein in *P. berghei* and another DHHC protein may be needed to compensate.

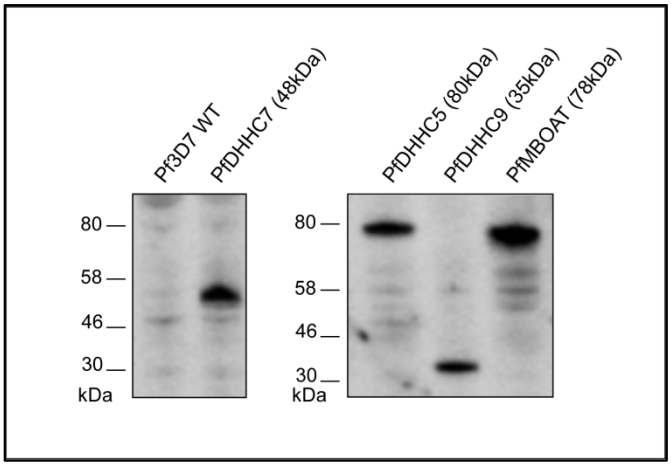


Figure 5.2: Expression of PfDHHC and PfMBOAT proteins in *P. falciparum* schizonts. Immunoblot analysis was performed on total protein lysates from saponin-lysed *P. falciparum* schizonts, expressing triple-HA-tagged DHHC and MBOAT proteins. Membranes were probed with α-HA antibodies and the expected protein sizes are shown in brackets.

5.1.3 Subcellular localisation of DHHC and MBOAT proteins in P. falciparum

In order to establish the subcellular localisation of these DHHC proteins in *P. falciparum,* immunofluorescence microscopy was performed on the parasite lines containing the 3-HA-tagged versions of the five PfDHHC proteins, as well as PfMBOAT, using antibodies against the 3-HA tag. Immunofluorescence signal was successfully detected for four of the PfDHHCs (PfDHHC3, 5, 7 and 9) as well as for PfMBOAT (Figure 5.3). Although the expression of PfDHHC3 was not detected by immunoblot, it was successfully detected by immunofluorescence. This suggests that the lack of detection by immunoblot (Section 5.1.2) might not be due to low expression levels, but could instead be due to solubility of the protein in the detergents used for protein extraction. Membrane proteins as a whole are difficult to extract due to their high hydrophobicity, and this is even truer for multi-TM-domain proteins, such as the DHHCs. Nevertheless, the successful detection of PfDHHC3 by immunofluorescence microscopy indicates that this protein is expressed in schizonts. Immunofluorescence signal was not detected for PfDHHC8, suggesting that the expression of PfDHHC8 is probably at a level that is too low to be detected by tagging at the endogenous locus. As the homologue of this protein in *P. berghei* (PbDHHC8, Section 4.2.3) was successfully detected by immunofluorescence, this further suggests that the expression levels of this DHHC might differ between the species.

Similar to what was observed in *P. berghei* (Section 4.2.3), the PfDHHC proteins, as well as PfMBOAT, appeared to be distributed to discrete foci in *P. falciparum* schizonts (Figure 5.3). The potential

subcellular localisations of all these proteins were determined by comparing their immunofluorescence signals to that of the following proteins with well-established localisations, for which high quality antibodies were available: ERD2 (Golgi marker) [4], BiP (ER marker) [5], rhoptry associated protein 1 (RAP1) (rhoptry marker) [6] and GAP45 (IMC marker) [7] (Figure 5.3).

The immunofluorescence staining of PfDHHC3 was compared to the staining of all five localisation markers described above. PfDHHC3 staining did not co-localise with BIP, GAP45, MSP1 or RAP1, indicating that PfDHHC3 was not localised at the ER, IMC, plasma membrane or the rhoptries. However, PfDHHC3 staining co-localised with ERD2 staining, indicating a Golgi localisation (Figure 5.3A). This was in contrast to its *P. berghei* homologue, PbDHHC3, which was localised to the IMC (Section 4.2.3). The difference in location of this DHHC protein between the two *Plasmodium* species could again be due to the fact that *P. berghei* is missing one DHHC protein, and thus may require another DHHC protein to compensate.

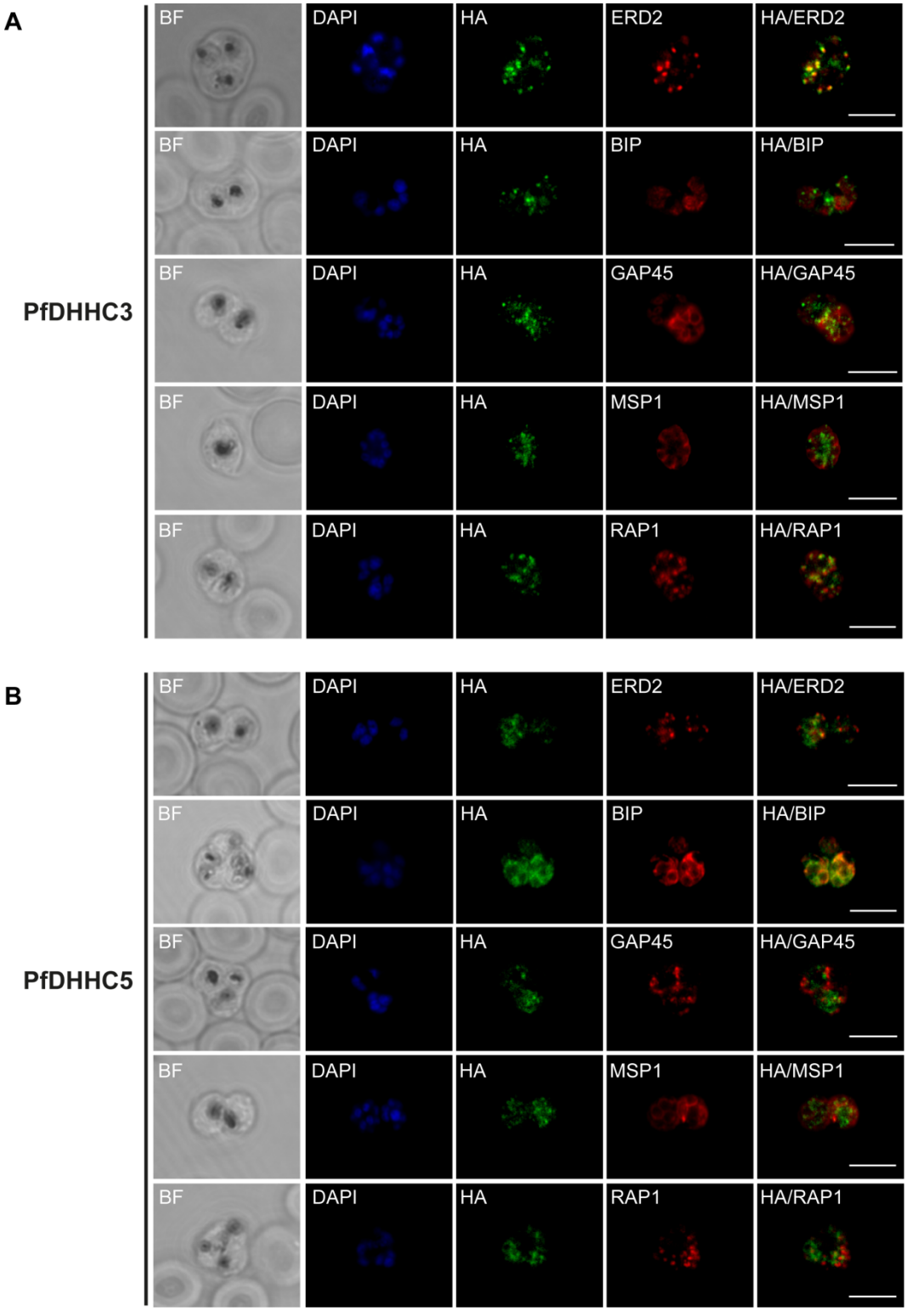
PfDHHC5 staining did not co-localise with ERD2, GAP45, MSP1 or RAP1 staining, but co-localised with BIP staining (Figure 5.3B), suggesting an ER localisation, and this was similar to the localisation of its *P. berghei* homologue, PbDHHC5 (Section 4.2.3).

PfDHHC7 displayed a punctate distribution that was not similar to that of ERD2 staining, indicating that it was not localised to the Golgi. PfDHHC7 also did not co-localise with BIP, GAP45 or MSP1, although staining with MSP1 indicated that PfDHHC7 appeared to be distributed apically. However, PfDHHC7 co-localised with RAP1 staining (Figure 5.3C), indicating a rhoptry localisation. PbDHHC7 staining was also punctate and was apically located in *P. berghei* schizonts (Section 4.2.3). That, and the fact that PfDHHC7 co-localises with RAP1, suggests that PbDHHC7 may also localise to the rhoptries.

PfDHHC9 did not co-localise with ERD2, BIP or RAP1 staining. However, PfDHHC9 appeared to co-localise with both GAP45 and MSP1 staining in late schizonts (Figure 5.3E). Further investigation by comparing PfDHHC9 staining to GAP45 and MSP1 staining in early schizonts, revealed that PfDHHC9 co-localised with GAP45 but not with MSP1 staining in the earlier stages of schizogony (Figure 5.3E). As described in the previous chapter (Section 4.2.3), IMC and plasma membrane staining is similar in mature schizonts but is distinctly different in early schizonts. Thus, this indicates an IMC localisation for PfDHHC9, which is in keeping with the localisation of its *P. berghei* homologue, PbDHHC9 (Section 4.2.3).

Lastly, PfMBOAT did not co-localise with ERD2, GAP45 or MSP1, indicating that it was not localised to the Golgi, IMC or plasma membrane. PfMBOAT did however co-localise with BIP staining (Figure 5.3D), indicating an ER localisation.

In summary, the five *Plasmodium* PATs studied here appear to be localised to different membrane compartments within the parasite. PfDHHC3 is localised to the Golgi, PfDHHC5 localises to the ER, PfDHHC7 localises to the rhoptries, PfDHHC9 localises to the IMC, and PfMBOAT also localises to the ER (Figure 5.3F). Similar to *P. berghei*, the *P. falciparum* PATs appear to be distributed both to organelles where PATs in other eukaryotic organisms are typically localised to, such as the ER and Golgi, as well as unique parasite organelles, such as the IMC and rhoptries. The results described here also indicate that although most of the homologues of the two *Plasmodium* species appear to be localised to the same place, this is not always the case, as PfDHHC3 and PbDHHC3 localised to different sites in the two *Plasmodium* species.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DAPI
HA
ERD2
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BF
DAPI
HA
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DAPI
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HA/GAP45
BF
DAPI
HA
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BIP
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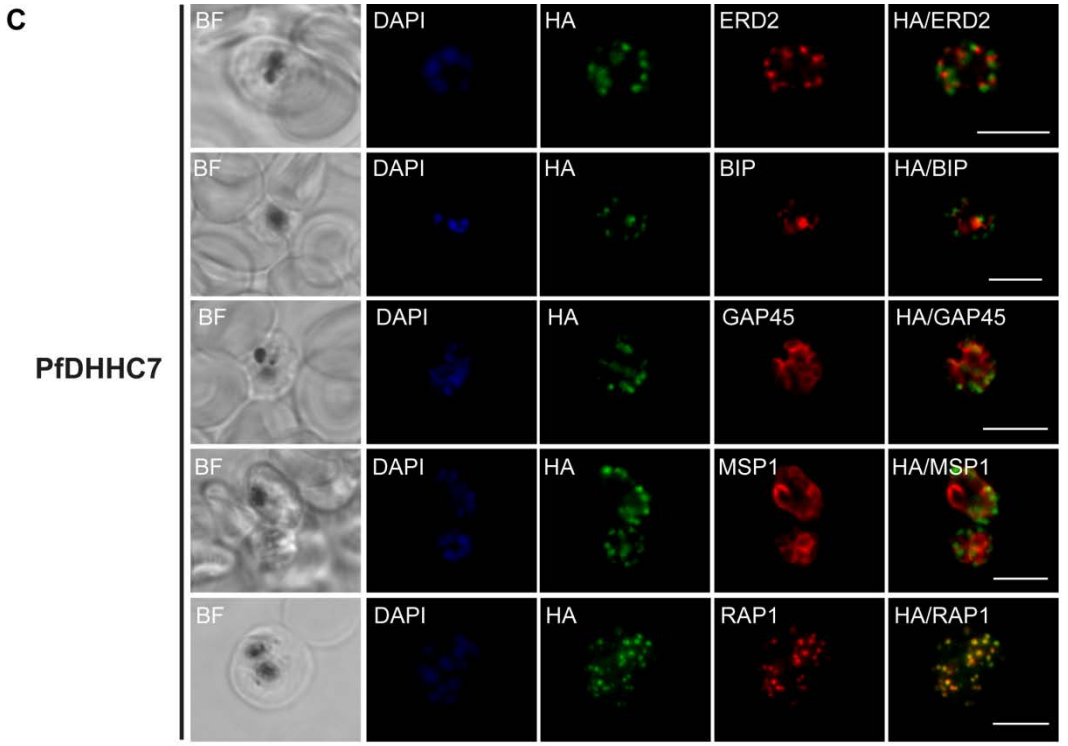
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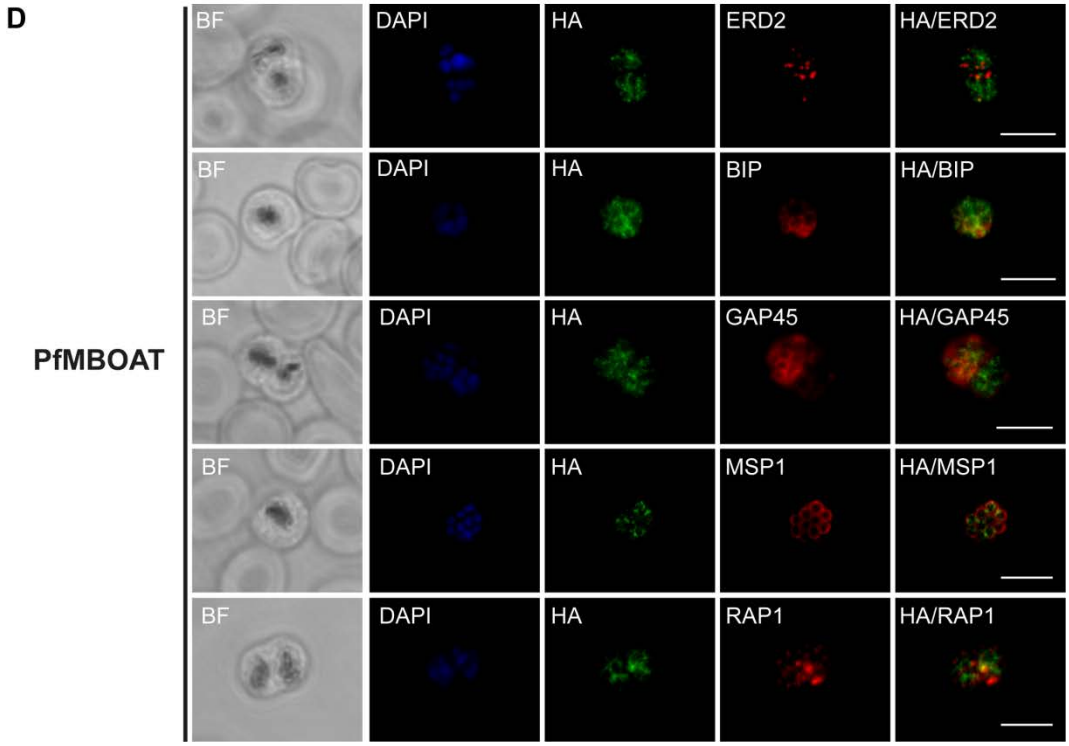
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PfMBOAT
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ERD2
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HA/MSP1
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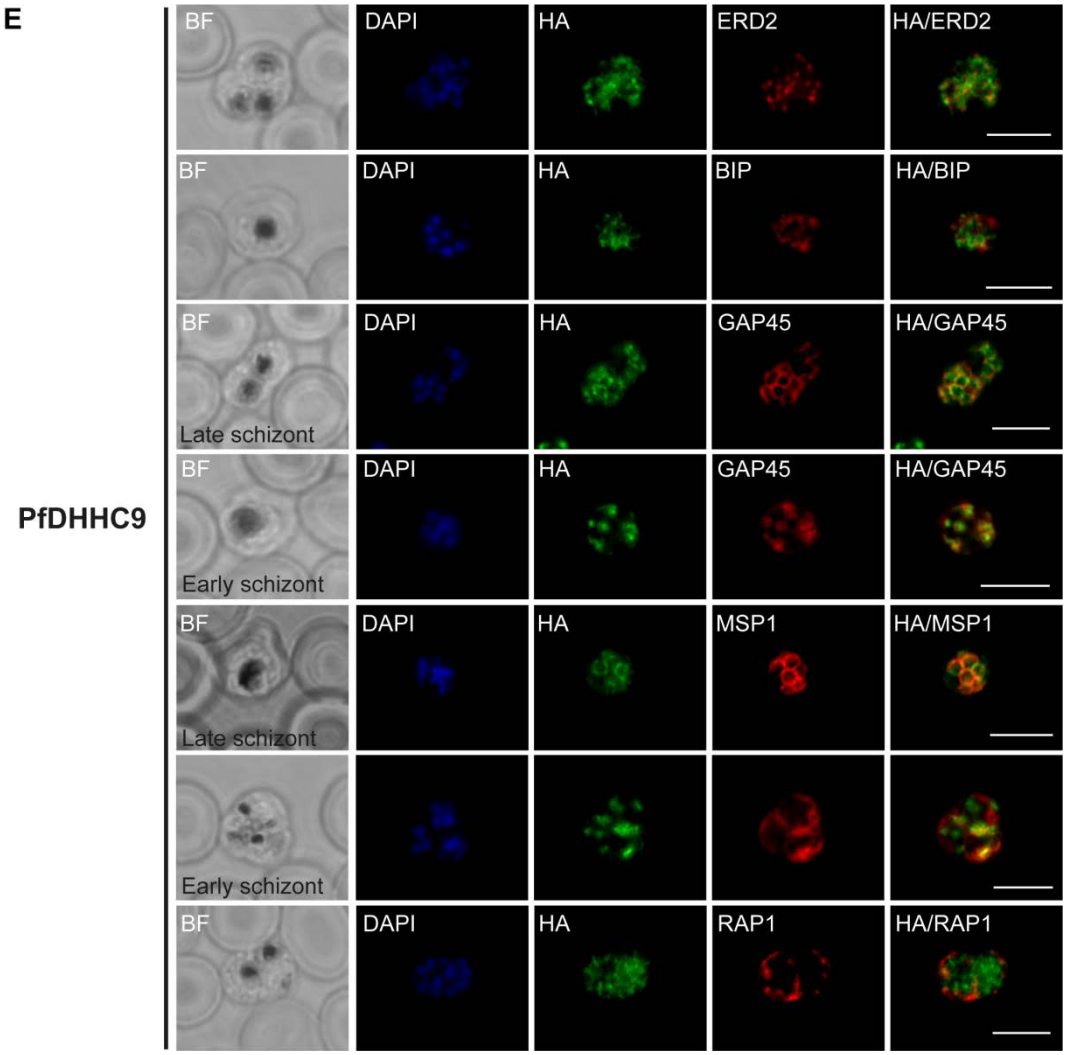
DAPI
HA
RAP1
HA/RAP1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PfDHHC9
BF
DAPI
HA
ERD2
HA/ERD2
BF
DAPI
HA
BIP
HA/BIP
BF
Late schizont
DAPI
HA
GAP45
HA/GAP45
BF
Early schizont
DAPI
HA
GAP45
HA/GAP45
BF
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HA
MSP1
HA/MSP1
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BF
DAPI
HA
RAP1
HA/RAP1

F

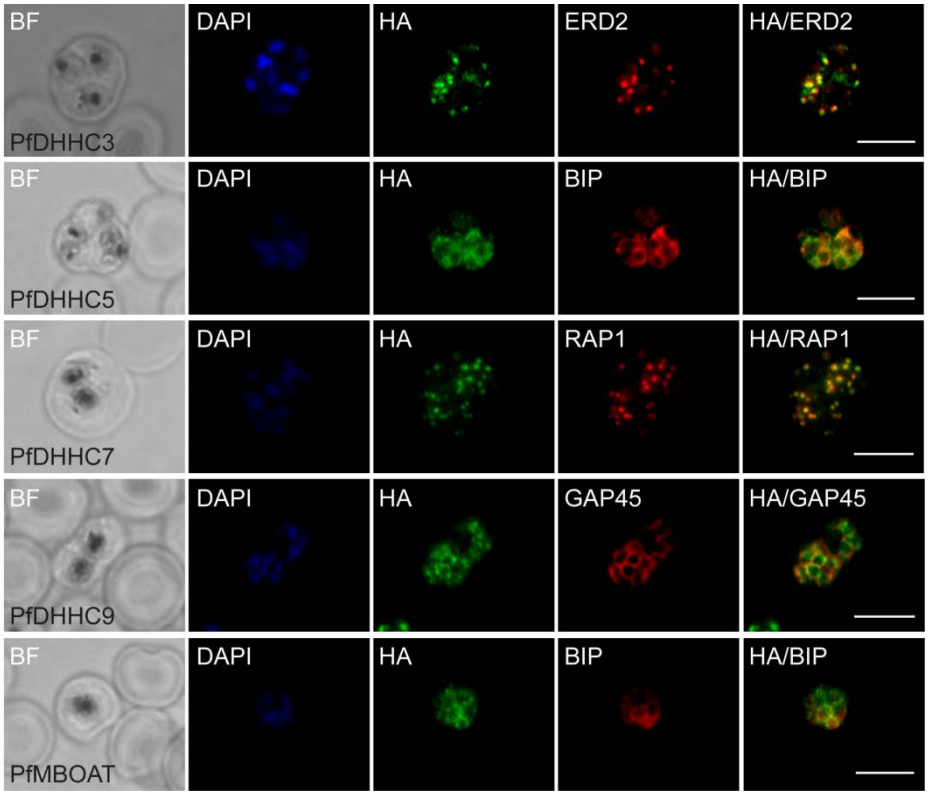


Figure 5.3: Localisation of DHHC and MBOAT proteins in *P. falciparum*. Triple-HA-tagged PfDHHC and PfMBOAT proteins were localised by immunofluorescence using antibodies against the 3-HA tag (green). Immunofluorescence staining of each of the tagged PfDHHC and PfMBOAT proteins were compared against that of the following known localisation markers (red): ERD2 (Golgi marker), BIP (endoplasmic reticulum marker), RAP1 (rhoptry marker) and GAP45 (inner membrane complex marker). Nuclear staining by DAPI is shown in blue. **(A)** Localisation of PfDHHC3. **(B)** Localisation of PfDHHC5. **(C)** Localisation of PfDHHC7. **(D)** Localisation of PfMBOAT. **(E)** Localisation of PfDHHC9. For the staining of PfDHHC9 with GAP45 and MSP1, both a late schizont, as well as an early schizont, is shown in order to differentiate between IMC and plasma membrane localisation. **(F)** Summary of the localisations of all PfDHHC and PfMBOAT proteins. Scale bar: 5 µm.

5.2. Essentiality of DHHC and MBOAT proteins in *P. falciparum*

5.2.1. Generation of PfDHHC and PfMBOAT knock-out transgenic parasite lines

In order to knock-out the PAT genes of interest (PfDHHC3, 5, 7, 8, and 9, and PfMBOAT), 600-800 bp of the N-terminal and C-terminal regions of each gene of interest were PCR amplified and introduced into the *P. falciparum* negative-selectable knock-out vector (pCC-1), which contains two selection cassettes: the human dihydrofolate reductase gene (*hDHFR)*, which confers resistance to the antifolate inhibitor WR99210 [8], and the *Saccharomyces cerevisiae* cytosine deaminase/uracil phosphoribosyltransferase chimeric gene (*ScFCU*), the 'suicide gene' which converts 5-fluorocytosine (5-FC) into the toxic 5-fluorouracil when 5-FC is present [9].

The two selection cassettes present in the pCC-1 knock-out vector allows two types of drug selection to be performed on transgenic parasites which have taken up the vector. Firstly, transgenic parasites are selected for using WR99210 (positive drug selection). The drug resistant parasite population, which consists of parasites which have integrated the construct into the genome, and parasites which are harbouring the construct episomally, can then be put through several rounds of drug cycling, where drug pressure is removed for 3 weeks before being reapplied. This is because episomal plasmids are unstable, and can be lost in the absence of drug pressure, whereas the stable integrant will be maintained [10]. Thus, the use of drug cycling should enrich for parasite populations which have integrated the construct and are not harbouring the construct episomally. Secondly, transgenic parasites containing the integrated construct can be further selected for using 5-FC (negative drug selection). In the presence of the episome, *ScFCU* is expressed and converts 5-FC to the toxic 5-fluorouracil. When the knock-out vector is integrated by double-crossover recombination, the episomal plasmid is linearised and *ScFCU* is not expressed, resulting in transgenic parasites which survive in the presence of 5-FC [9]. Unfortunately, the use of 5-FC negative selection does not select against single-crossover recombination, as in this form of recombination, the entire plasmid is inserted into the genome and *ScFCU* is inserted into the endogenous locus in a 3' to 5' configuration, with its promoter also in a 3' to 5' configuration, and is thus also not expressed.

The *P. falciparum* PAT knock-out constructs were designed such that the N-terminal and C-terminal targeting regions of each gene were inserted into the 5' and 3' flanking regions of the *hDHFR* selection cassette respectively, as shown in Figure 5.4A and described in Materials and Methods. These PfDHHC and PfMBOAT knock-out vectors were then introduced into *P. falciparum* strain 3D7 using the transfection methods described in Materials and Methods. After transfection and positive drug selection using WR99210, as well as after drug cycling, transgenic parasites that had taken up

the knock-out constructs and thus gained resistance to WR99210 were obtained for all six PAT genes studied here (PfDHHC3, 5, 7, 8 and 9, and PfMBOAT). The genomic DNA of all six transgenic parasite lines were then genotyped using PCR amplification in order to determine whether the knock-out construct had been integrated into the genomic DNA or was being maintained episomally.

5.2.2. Genotyping of PfDHHC and PfMBOAT knock-out transgenic parasite lines

The integration of the PfDHHC and PfMBOAT knock-out constructs into the genomic DNA was expected to occur via double-crossover recombination at the homologous N-terminal and C-terminal targeting regions. This would result in the replacement of a section of the genes of interest –which includes the conserved DHHC domain (for the PfDHHC proteins) or the conserved histidine residue (for PfMBOAT protein) - with the *hDHFR* selection cassette, thus causing the disruption of the genes of interest, as shown in Figure 5.4A.

Integration of these knock-out constructs could also potentially occur via single-crossover recombination at either the N-terminal or the C-terminal homologous targeting regions (Figure 5.4A). In this case, instead of just the *hDHFR* selection cassette, the entire plasmid is inserted into the endogenous locus. As mentioned above, treatment with the negative selection drug, 5-FC, would not select against this form of integration, as in both cases, *ScFCU* is inserted into the genome in a 3' to 5' configuration and is thus not expressed. With single-crossover recombination, although the gene of interest is still disrupted, a complete copy of the gene is still present. However, if the single-crossover recombination were to occur at the N-terminal region, the complete copy of the gene is moved further downstream, away from its endogenous promoter (Figure 5.4A), and expression of the wild-type gene could potentially be reduced.

As mentioned above, after transfection and positive drug selection, transgenic parasites which had taken up the knock-out constructs were obtained for all six PAT genes studied here. The genomic DNA of all six transgenic parasites lines were analysed by PCR amplification for the presence of sequences specific to the integrated construct –both double-crossover and single crossover (N-terminal and C-terminal) - the wild-type locus and the episomal plasmid, using the primer pairs shown in the schematic in Figure 5.4A and listed in the table in Figure 5.4B and in Table 2.6.

(1) Genotyping of PfDHHC3 and 7, and PfMBOAT knock-out transgenic parasite lines

For these 3 transgenic strains, analysis by PCR amplification revealed the presence of sequences specific to the wild-type locus (primer pair: P6+P10) and the episome (primer pair: P8+P9). No sequences specific to the double-crossover integrated construct (primer pair:

P6+P9), N-terminal single-crossover integrated construct (primer pair: P8+P10) or C-terminal single-crossover integrated construct (primer pair: P12+P13) were observed (Figure 5.4B). Furthermore, amplification of the full-length gene locus (primer pair: P6+P7) resulted in a fragment with a size equal to the expected size of the wild-type locus (Figure 5.4B). This was still the case even after drug cycling. The three transgenic lines were then treated with the negative selection drug, 5-FC, in an attempt to remove parasites harbouring the plasmid episomally, with the intention to select for the parasite population which contained the integrated construct, if such a population existed. Strangely, sequence for the episome was still amplified even after 5-FC treatment, indicating that the episome still remained in the presence of 5-FC. This was observed even in increased concentrations of 5-FC. The reason for this is unknown. However, a potential explanation could be that the PfDHHC3 and 7 and PfMBOAT genes are not amenable to disruption, but the transgenic parasites have somehow gained resistance to 5-FC in an attempt to survive. A similar phenotype, where 5-FC resistance was gained without loss of the *ScFCU*-containing plasmid has also been previously observed to occur in *P. falciparum* [11], and indeed, spontaneous resistance to 5-FC has been documented in other species [12, 13]. Given that there was no evidence of integration for any of these 3 strains, they were not analysed any further.

(2) Genotyping of PfDHHC8 knock-out transgenic parasite line

PCR analysis of this transgenic line resulted in the amplification of sequences specific to the wild-type locus (primer pair: P6+P10) and the episome (primer pair: P8+P9). No sequence was amplified for the double-crossover integrated construct (primer pair: P6+P9), N-terminal single-crossover integrated construct (primer pair: P8+P10), or the C-terminal single-crossover integrated construct (primer pair: P12+P13) (Figure 5.4B). Amplification of the full-length gene locus (primer pair P6+P7) resulted in a fragment with a size equal to that expected for the wild-type locus (Figure 5.4B). This was still observed after drug cycling. However, in this case, subsequent negative selection with 5-FC resulted in parasite death and no parasites were observed even after three weeks of culturing. This could be an indication that the PfDHHC8 gene cannot be disrupted, and suggests that PbDHHC8 could thus be an essential gene. As with PfDHHC3 and 7, and PfMBOAT transgenic lines, the PfDHHC8 transgenic line was not analysed any further.

(3) Genotyping of PfDHHC5 knock-out transgenic parasite line

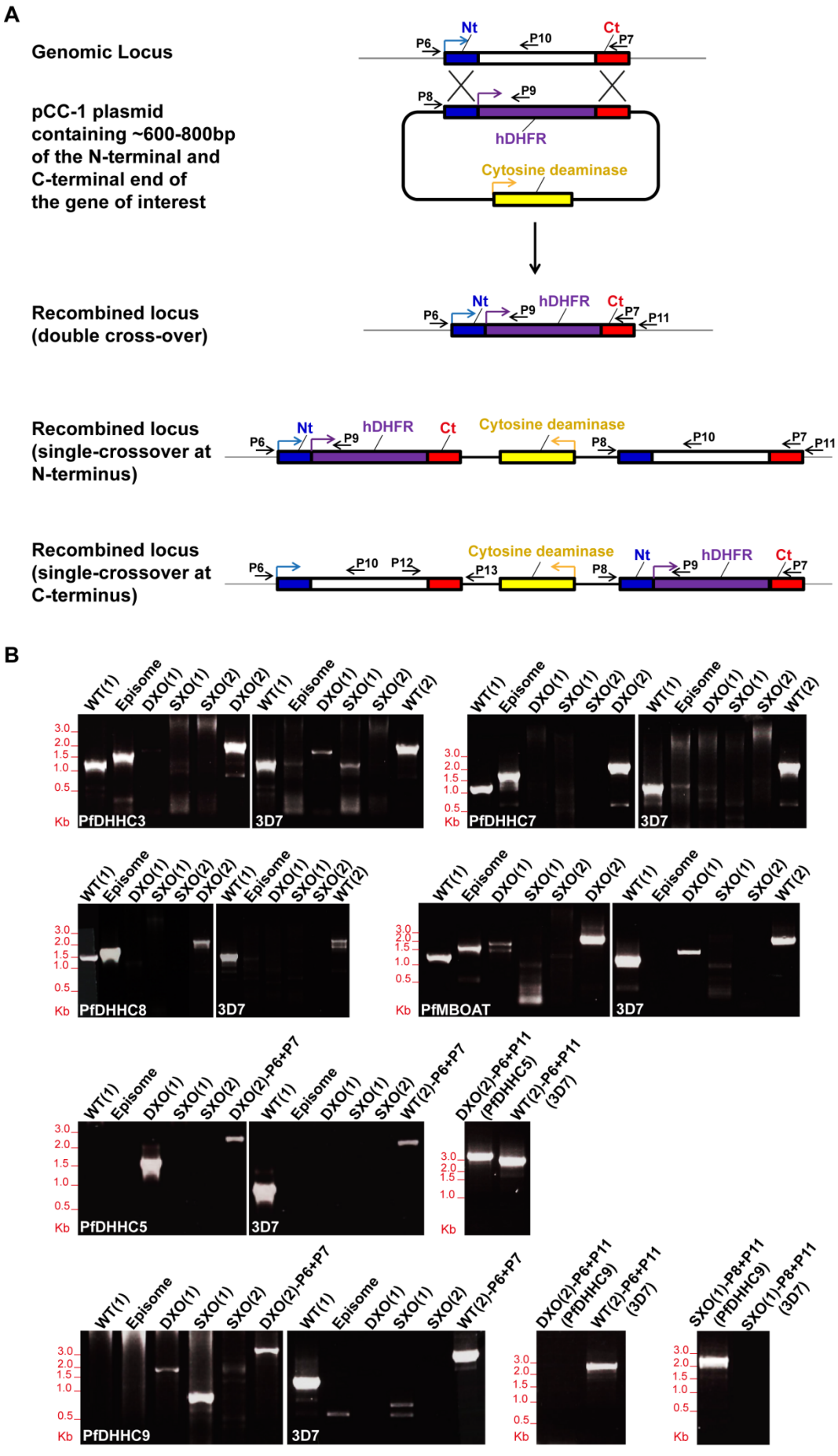
After positive drug selection, drug cycling, as well as negative selection with 5FC, PCR analysis of this transgenic line revealed the presence of sequences specific to the double-crossover integrated construct (primer pair P6+P9). Sequences specific to the wild-type locus (primer pair: P6+P10) and the episome (primer pair P8+P9) were no longer amplified (Figure 5.4B), and no amplification was observed for the N-terminal single-crossover integrated construct (primer pair P8+P10) or the C-terminal single-crossover integrated construct (primer pair: P12+P13). Amplification of the full-length gene locus (primer pair: P6+P7) resulted in a fragment with a size equal to the size expected if double-crossover integration had occurred (Figure 5.4B). This was further confirmed using an additional primer (primer P11) located in the 3' UTR of the PfDHHC5 gene (Figure 5.4A). Amplification of the full-length gene locus using primer P6 with primer P11 also resulted in a fragment with a size equal to the size expected if double-crossover integration had occurred (Figure 5.4B). This indicated that for this transgenic line, double-crossover recombination had occurred, and the PfDHHC5 gene had been disrupted, resulting in a PfDHHC5 knock-out transgenic line. The PfDHHC5 knock-out line was then cloned by limiting dilution, as described in Materials and Methods, in order to generate parasite clones derived from single parasites. All clones were also analysed by PCR in order to ensure that each clonal population displayed double-crossover integration (data not shown).

(4) Genotyping of PfDHHC9 knock-out transgenic parasite line

After positive drug selection, drug cycling and negative selection with 5FC, PCR analysis revealed the presence of sequence specific to single-crossover integration at the N-terminus (primer pair: P8+P10) (Figure 5.4B). Sequence specific to the episome (primer pair: P8+P9) and the unmodified wild-type locus (primer pair: P6+P10) were no longer amplified, indicating that the episome was not retained and that integration had indeed occurred (Figure 5.4B). Sequence specific to the C-terminal single-crossover integrated construct (primer pair: P12+P13) was also not amplified (Figure 5.4B). Amplification of sequence indicating double-crossover integration (primer pair: P6+P9) was observed, and amplification of the full-length gene locus (primer pair: P6+P7) resulted in a fragment with a size equal to the size expected if double-crossover integration had occurred. However, the use of both these double-crossover integration primer pairs described above would result in amplification of fragments which also exist in an N-terminal single-crossover integrated construct, as shown in the schematic in Figure 5.4A. Therefore, further analysis by PCR was

performed using an additional primer located in the 3' UTR of the PfDHHC9 gene (primer P11, Figure 5.4A and B). Amplification of the full-length gene locus using primer P6 with primer P11 did not amplify either the fragment expected if double-crossover recombination had occurred, or the fragment expected for the wild-type locus (Figure 5.4B). However, sequence specific to the presence of the wild-type locus located downstream of the inserted knock-out construct (primer pair: P8+P11) was amplified instead (Figure 5.4B), confirming that the knock-out vector had indeed integrated via single-crossover at the N-terminus. This means that although the PfDHHC9 gene has been disrupted, a full-length copy of the wild-type gene is still present. However, as the wild-type copy has now moved away from its endogenous promoter, expression of the gene could potentially be reduced, perhaps resulting in a knock-down rather than a knock-out line. Again, the PfDHHC9 transgenic line was cloned by limiting dilution and all clones analysed by PCR (data not shown).

The genotyping of all the transgenic parasite strains described above indicated that although transfection of the knock-out constructs was successful and parasites resistant to WR99210 were obtained for all six PATs, successful integration was detected for only two of the proteins of interest (PfDHHC5 and 9). It must be noted however, that all the genotyping described here was performed by PCR analysis and further confirmation of the integration of these knock-out constructs should be also performed by Southern blot analysis. Unfortunately, due to time constraints, data from Southern blot analysis was not yet available at the time of writing this dissertation.


A
Genomic Locus
pCC-1 plasmid containing ~600-800bp of the N-terminal and C-terminal end of the gene of interest
Recombined locus (double cross-over)
Recombined locus (single-crossover at N-terminus)
Recombined locus (single-crossover at C-terminus)
Nt
Ct
hDHFR
Cytosine deaminase
P6
P7
P8
P9
P10
P11
P12
P13
B
WT(1)
Episome
DXO(1)
SXO(1)
SXO(2)
DXO(2)
WT(2)
DXO(2)-P6+P7
WT(2)-P6+P7
DXO(2)-P6+P11 (PfDHHC5)
WT(2)-P6+P11 (3D7)
DXO(2)-P6+P11 (PfDHHC9)
SXO(1)-P8+P11 (PfDHHC9)
SXO(1)-P8+P11 (3D7)
Kb
3.0
2.0
1.5
1.0
0.5
PfDHHC3
3D7
PfDHHC7
PfDHHC8
PfMBOAT
PfDHHC5
PfDHHC9

Knock-out line	Sequence amplified	Primer pair	Expected Size (bp)
PfDHHC3	WT(1) - Wild-type (Fragment)	P6+P10	1294
	WT(2) - Wild-type (Full-length)	P6+P7	2291
	Episome	P8+P9	1769
	DXO(1) - Double-crossover integration (Fragment)	P6+P9	1978
	DXO(2) - Double-crossover integration (Full-length)	P6+P7	3727
	SXO(1) - Single-crossover integration (Nt)	P8+P10	1050
	SXO(2) - Single-crossover integration (Ct)	P12+P13	1075
PfDHHC5	WT(1) - Wild-type (Fragment)	P6+P10	923
	WT(2) - Wild-type (Full-length)	P6+P7 and P6+P11	2753 and 2908
	Episome	P8+P9	1794
	DXO(1) - Double-crossover integration (Fragment)	P6+P9	1780
	DXO(2) - Double-crossover integration (Full-length)	P6+P7 and P6+P11	3462 and 3617
	SXO(1) - Single-crossover integration (Nt)	P8+P10	920
	SXO(2) - Single-crossover integration (Ct)	P12+P13	1907
PfDHHC7	WT(1) - Wild-type (Fragment)	P6+P10	1268
	WT(2) - Wild-type (Full-length)	P6+P7	2369
	Episome	P8+P9	1801
	DXO(1) - Double-crossover integration (Fragment)	P6+P9	1918
	DXO(2) - Double-crossover integration (Full-length)	P6+P7	3596
	SXO(1) - Single-crossover integration (Nt)	P8+P10	1151
	SXO(2) - Single-crossover integration (Ct)	P12+P13	1903
PfDHHC8	WT(1) - Wild-type (Fragment)	P6+P10	1468
	WT(2) - Wild-type (Full-length)	P6+P7	2769
	Episome	P8+P9	1755
	DXO(1) - Double-crossover integration (Fragment)	P6+P9	2036
	DXO(2) - Double-crossover integration (Full-length)	P6+P7	3765
	SXO(1) - Single-crossover integration (Nt)	P8+P10	1153
	SXO(2) - Single-crossover integration (Ct)	P12+P13	1380
PfDHHC9	WT(1) - Wild-type (Fragment)	P6+P10	1342
	WT(2) - Wild-type (Full-length)	P6+P7 and P6+P11	2406 and 2539
	Episome	P8+P9	1651
	DXO(1) - Double-crossover integration (Fragment)	P6+P9	1992
	DXO(2) - Double-crossover integration (Full-length)	P6+P7 and P6+P11	3711 and 3844
	SXO(1) - Single-crossover integration (Nt)	P8+P10 and P8+P11	1001 and 2168
	SXO(2) - Single-crossover integration (Ct)	P12+P13	1144
PfMBOAT	WT(1) - Wild-type (Fragment)	P6+P10	1190
	WT(2) - Wild-type (Full-length)	P6+P7	2414
	Episome	P8+P9	1684
	DXO(1) - Double-crossover integration (Fragment)	P6+P9	1979
	DXO(2) - Double-crossover integration (Full-length)	P6+P7	3755
	SXO(1) - Single-crossover integration (Nt)	P8+P10	895
	SXO(2) - Single-crossover integration (Ct)	P12+P13	1473

Figure 5.4: Generation of PfDHHC and PfMBOAT knock-out transgenic lines in *P. falciparum*. (A) Scheme of the strategy used to knock-out the endogenous locus of the PfDHHC and PfMBOAT genes of interest. The primer positions illustrated here indicate the primers used for genotyping of the PfDHHC and PfMBOAT knock-out transgenic lines. Flags indicate promoter regions. **(B)** Genotyping of the PfDHHC and PfMBOAT knock-out transgenic lines by genomic PCR analysis. Primer pairs used for the amplification of sequences specific to the wild-type locus, episome and integrated (double crossover and single crossover) constructs are as listed in the table, along with the expected sizes of the fragments. For PfDHHC5 and PfDHHC9 knock-out transgenic lines, additional PCR analysis using the primer P11 was used to confirm integration of the construct.

5.2.3. Analysis of gene expression in PfDHHC5 and 9 knock-out transgenic parasite lines

In order to confirm that the expression of PfDHHC5 and 9 proteins was abolished or at least reduced in the PfDHHC5 and 9 knock-out lines, RNA was extracted from blood-stage cultures infected with the transgenic lines of interest and reverse transcribed to make cDNA, as described in Materials and Methods. This was done for three of the limiting dilution clones of each knock-out/knock-down line: PfDHHC5 clones 6, 8, and 19, and PfDHHC9 clones 1, 2, and 3, as well as for wild-type 3D7 as a control. The presence of sequences specific to PfDHHC5 and 9 within the cDNA of all parasite lines was tested by PCR amplification, using the primer pairs listed in Table 2.7. No amplification was observed in the cDNA of all three PfDHHC5 transgenic clones when using primers specific for sequences within the PfDHHC5 gene (Figure 5.5A), indicating that PfDHHC5 is not expressed and the PfDHHC5 clones are indeed knock-out clones. For the cDNA of all three PfDHHC9 clones, reduced PCR amplification was observed when using primers specific for sequences within the PfDHHC9 gene (Figure 5.5B), indicating a reduced expression of PfDHHC9 protein. This suggests that the PfDHHC9 clones, although not truly knocked-out, are at least knock-down lines.

Further analysis by quantitative PCR (qPCR) was performed using the cDNA from one clone of each PfDHHC knock-out line (PfDHHC5 clone 19 and PfDHHC9 clone 3, along with wild-type 3D7 as a control). Primers and 6-carboxyfluoroscein (FAM)-labelled probes that were specific for each PfDHHC gene were used in the qPCR reaction, as described in the Materials and Methods (the qPCR experiment described here was kindly performed by Leyla Bustamente, Rayner lab). The qPCR analysis revealed that the expression of the PfDHHC5 gene was completely abolished in the PfDHHC5 clone 19 transgenic line (Figure 5.5C). Meanwhile, although some expression of the PfDHHC9 gene could still be detected, expression was greatly reduced in the PfDHHC9 clone 3 transgenic line (Figure 5.5C). Taken together, this indicated that PfDHHC5 was successfully knocked-out, while PfDHHC9 was knocked down, in *P. falciparum*.

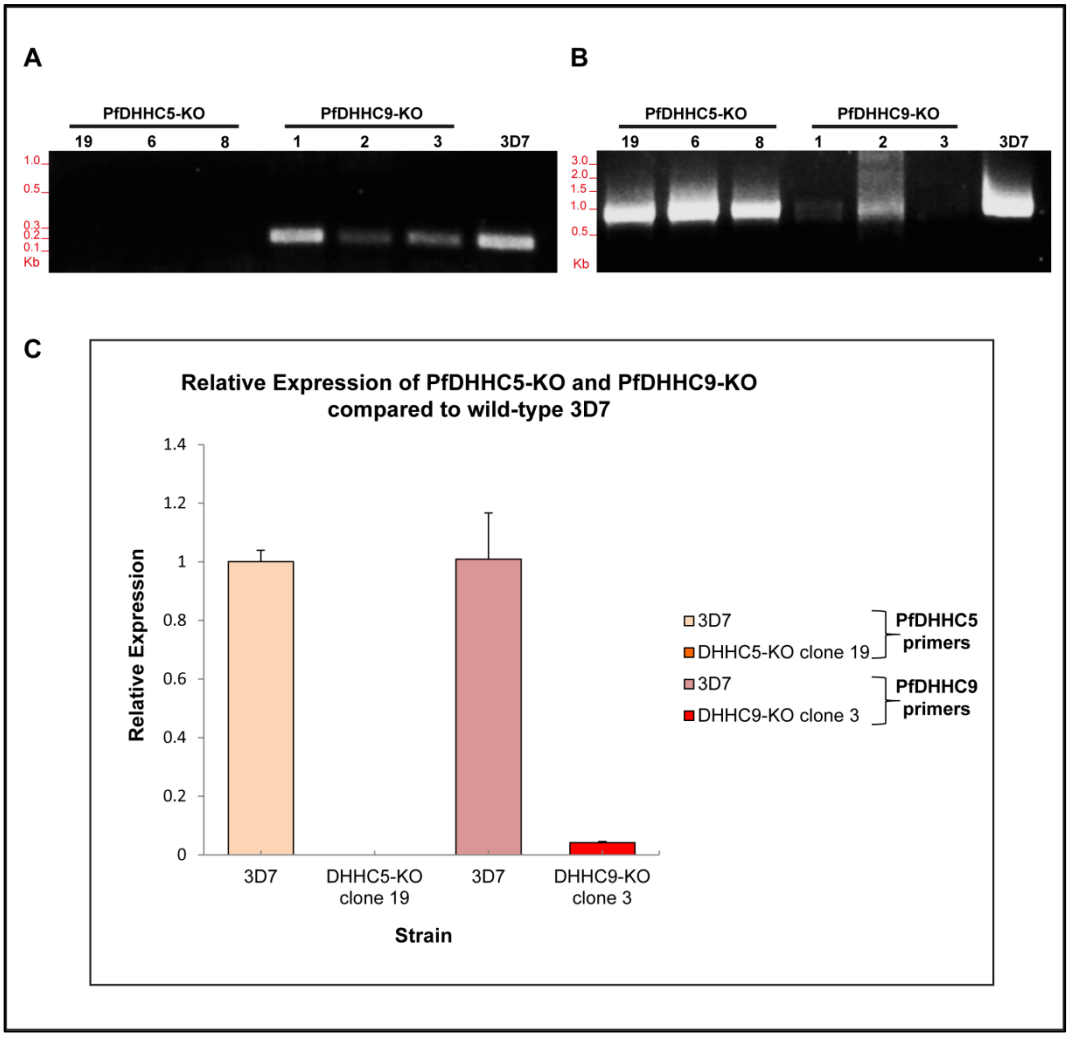


Figure 5.5: Reverse transcription (RT)-PCR analysis of PfDHHC5-KO and PfDHHC9-KO transgenic lines. cDNA was made by RT-PCR for PfDHHC5-KO clones (19, 6, and 8) and PfDHHC9-KO clones (1, 2, and 3), as well as for wild-type 3D7. Sequence specific to each PfDHHC gene was amplified by PCR using the cDNA of all clones, along with wild-type 3D7. **(A)** The primers used amplify a sequence specific to the PfDHHC5 gene. **(B)** The primers used amplify a sequence specific to the PfDHHC9 gene. **(C)** Quantitative PCR (qPCR) analysis of PfDHHC5-KO clone 19 and PfDHHC9-KO clone 3 relative to wild-type 3D7, using primers and 6-FAM-labelled probes specific to each gene.

5.2.3. Effect of the knock-out/knock-down of PfDHHC5 and 9 on parasite growth

The results described above indicate that of the six transgenic lines made, only PfDHHC5 and 9 transgenic parasite lines had integrated the knock-out constructs, and were disrupted at the genes of interest, with completely abolished expression for PfDHHC5 and reduced expression for PfDHHC9. For ease of description, both PfDHHC5 and 9 transgenic lines are now referred to as PfDHHC5-KO and PfDHHC9-KO. In order to determine the effect of disrupting these DHHC proteins on parasite growth, a growth assay was set up for both PfDHHC5-KO and PfDHHC9-KO as described in Materials and Methods. The growth assay was performed on three PfDHHC5-KO clones (6, 8, and 19) and three PfDHHC9-KO clones (1, 2, and 3), as well as on wild-type 3D7 as a control.

Briefly, the parasitemia of all knock-out parasite lines, including the wild-type 3D7 control, was measured by flow cytometry (with the kind assistance of Michel Theron, Rayner lab) every 2 days, over a period of 10 days. The growth of the PfDHHC5-KO and PfDHHC9-KO clones was then compared to that of wild-type 3D7. For both PfDHHC5-KO and PfDHHC9-KO, there was no significant difference in growth compared to wild-type 3D7 (Figure 5.6), indicating that the trangenic parasites appeared to be able to survive and grow normally in the absence of PfDHHC5 and with reduced PfDHHC9.

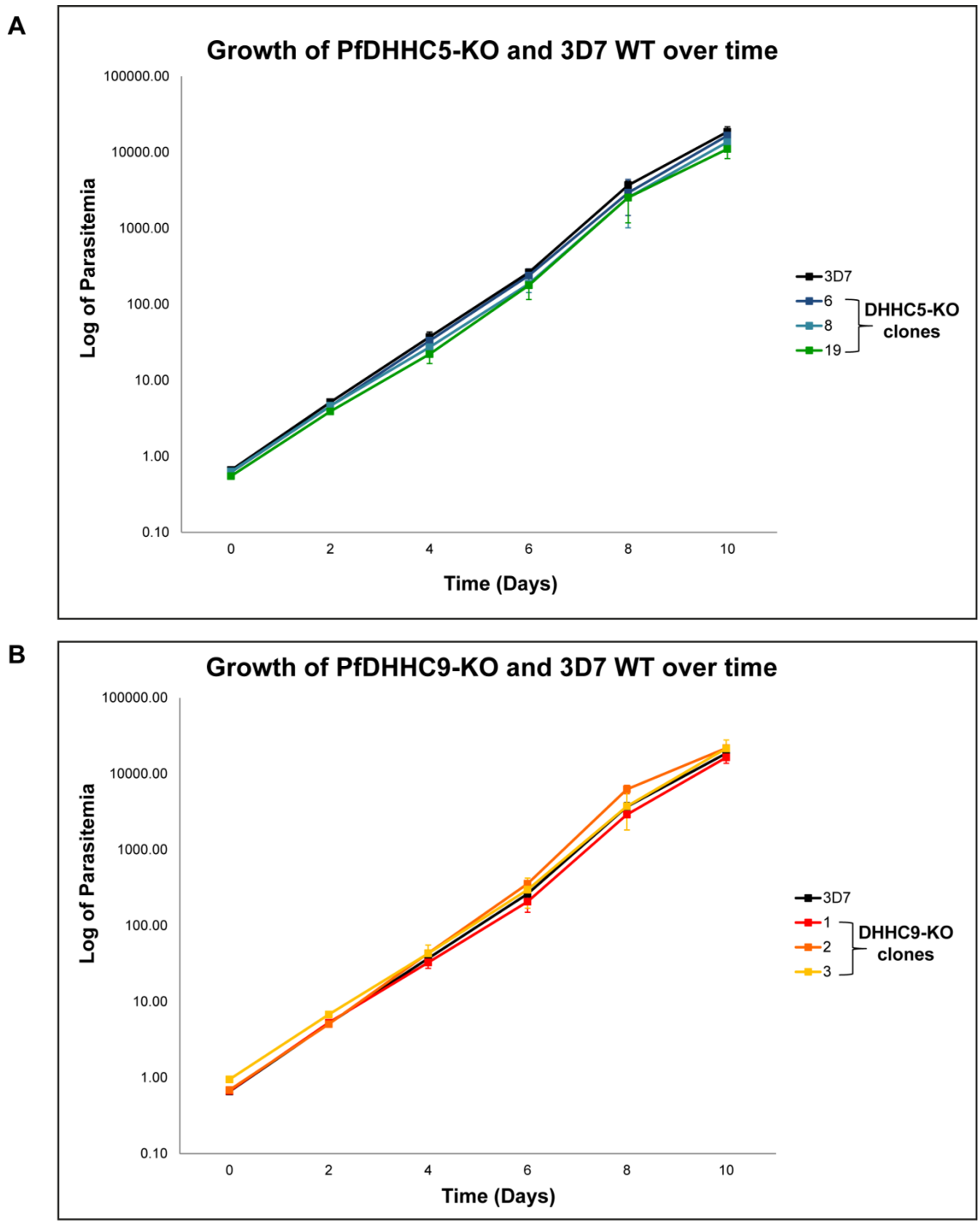


Figure 5.6: Growth assay comparing the growth of PfDHHC5-KO and PfDHHC9-KO with wild-type 3D7. (A) Parasitemia of PfDHHC5-KO clones (6, 8, and 19), along with wild-type 3D7, measured every 2 days for a total of 10 days. **(B)** Parasitemia of PfDHHC9-KO clones (1, 2, and 3) along with wild-type 3D7 measured every 2 days for a total of 10 days.

In summary, of the five *P. falciparum* DHHC-domain-containing proteins analysed here, only two proteins, namely PfDHHC5 and 9, were successfully disrupted, and disruption of these proteins appeared to have no detrimental effect on parasite growth and survival in parasite blood stages. However, it must be noted that further phenotyping of the knock-out strains generated here was unable to be performed due to time constraints. The remaining DHHC proteins were unable to be disrupted, at least in the intraerythrocytic stages studied here.

This implies that a subset of the *P. falciparum* DHHC proteins appear to be amenable to disruption in the intraerythrocytic stages, suggesting a possible functional redundancy for some of the PfDHHC proteins. This was similar to *P. berghei* DHHC proteins, where PbDHHC5 and 9 were also successfully disrupted in the blood stages (Section 4.3), just as PfDHHC5 and PfDHHC9 could be disrupted in *P. falciparum* blood stages. Conversely, some of the *P. falciparum* DHHC proteins were unable to be disrupted in the intraerythrocytic stages, suggesting that some DHHC proteins could be essential for *P. falciparum* blood-stage growth. This was again similar to *P. berghei*, where PbDHHC8 was unable to be disrupted in the blood stages (Section 4.3), just as PfDHHC8 could not be disrupted in *P. falciparum*.

Interestingly, PfDHHC3 and 7 were unable to be disrupted in *P. falciparum*, despite successful disruption in *P. berghei* (Section 4.3). This could possibly be due to the more efficient genetic manipulation techniques available in *P. berghei*, as well as superior gene targeting vectors [14], which greatly improve the efficiency of genetic modification in this parasite. On the other hand, this could also be truly a difference in gene function between the two *Plasmodium* species, again because of the difference in the total number of DHHC proteins between the two species, as well as the difference in localisation of some of the DHHC proteins.

In the case of PfMBOAT, the PfMBOAT coding gene was unsuccessfully disrupted here. Other groups have reported similar findings [1], suggesting that PfMBOAT may be essential in the intraerythrocytic stages. PfMBOAT is actually annotated on *Plasmo*DB to act as a diacylglycerol acyltransferase (DGAT), a common activity performed by members of the MBOAT family, and other work has shown that PfMBOAT does in fact exhibit DGAT activity [1]. Whether or not PfMBOAT also acts as a PAT is still unknown. However, the fact that it does play a role as a DGAT, which is an important enzyme in the biosynthetic pathway of triacylglycerol, may be the reason why this gene does not appear to be amenable to disruption.

Conclusion

This analysis of PAT proteins in *P. falciparum* has revealed similar insights to that of the study of these proteins in *P. berghei.* Firstly, as in *P. berghei*, *P. falciparum* DHHC proteins are localised to different membrane-bound sites in the parasite, including specialised parasite organelles. Secondly, some of the DHHC proteins in *P. falciparum* could possibly be essential for blood-stage growth, while other DHHC proteins appear to have functional redundancy. However, there appears to be some differences in the localisation and essentiality between some of the homologues of these DHHC proteins in the two *Plasmodium* species. Nevertheless, the fact that these DHHC proteins may play an important role in *Plasmodium* biology seems apparent. However, whether these proteins do in fact exhibit PAT activity is still unknown, and is addressed in the next chapter.

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