Chapter 4

Protein acyltransferases in *Plasmodium berghei*

4.1. Repertoire of DHHC-domain-containing proteins in *Plasmodium*

In order to achieve the aim of characterising the putative PATs in *Plasmodium*, it was first necessary to identify the repertoire of DHHC-domain-containing proteins in both *Plasmodium* species of interest here, *P. falciparum* and *P. berghei*. Search by protein-protein BLAST (blastp), using the 50 amino acid characteristic conserved DHHC cysteine-rich domain of the yeast DHHC protein, Erf2, revealed 12 proteins containing the conserved DHHC-domain in *P. falciparum,* all except one possessing a homologue in *P. berghei*, which has 11 proteins with the DHHC-domain. All of these putative DHHC proteins in both *P. falciparum* and *P. berghei* adhered to the generic structure of DHHC-PATs (shown in Figure 1.2), containing at least four TM-domains and possessing the DHHC-cysteine rich domain within an inter-TM-domain loop. For both *Plasmodium* species, each had one protein which contained a DHYC rather than the characteristic DHHC motif. This is similar to one of the yeast DHHC proteins, Akr1, which also contains the DHYC motif but is known to function as a DHHC-PAT [1]. A multiple sequence alignment of the amino acid sequences of the DHHC-cysteine rich regions of all these proteins across *P. falciparum* and *P. berghei* confirmed that this signature motif was indeed highly conserved in all the DHHC-containing proteins in both *Plasmodium* species, as shown in Figure 4.1A. The two other motifs usually conserved in DHHC proteins, the DPG and TTxE motifs, are also present in most of the *Plasmodium* DHHC proteins, as indicated in Table 4.1.

Phylogenetic analysis using neighbour-joining (NJ) [2] and maximum likelihood (ML) [3] methods on the conserved DHHC domains of *P. falciparum* and *P. berghei,* and the related Apicomplexan parasites, *Toxoplasma gondii* and *Neospora caninum,* revealed that an evolutionary relationship does exist between the DHHC proteins of these Apicomplexan parasites. For *P. falciparum* and *P. berghei*, all the DHHC proteins appeared to be grouped in orthologous pairs, except for the one *P. falciparum* DHHC protein (PFB0140w) which did not have a homologue in *P. berghei*. Although homologues of PFB0140w were present in other human *Plasmodium* species, there were no homologues of this protein in all rodent *Plasmodium* species, and PFB0140w did not group with the other Apicomplexan DHHC-domain-containing proteins, suggesting that this protein may have been gained in human and closely related simian *Plasmodium* species. Five of the DHHC proteins in *P. falciparum* and *P. berghei* appeared to have direct one-to-one orthologues in *T. gondii.* The rest of the *Plasmodium* DHHC proteins had related DHHC proteins in *T. gondii*, but clear one-to-one orthologues could not be identified as *T. gondii* has an expanded DHHC protein family of 18 proteins. A phylogenetic tree was generated from this data using both the NJ distance analysis and the ML analysis, and is shown in Figure 4.1B, where only nodes with a bootstrap value more than 80 are indicated and values of more than 95 are considered significant. The phylogenetic analysis described here was performed by Arnault Graindorge, Soldati-Favre lab as part of a collaborative project on *P. berghei* and *T. gondii*

DHHCs which was recently published in Traffic [4]. Based on these homology relationships, the *Plasmodium* DHHC proteins have now been named according to their closest *T. gondii* homologue, while preserving the orthology between *P. falciparum* and *P. berghei* pairs. The naming of the *Plasmodium* DHHC proteins along with their accession numbers is as shown in Table 4.1.

Plasmodium berghei			*Plasmodium falciparum*		
Name	Plasmodb accession	C-terminal motif	Name	Plasmodb accession	C-terminal motif
PbDHHC1	PBANKA_040200	NPG; TFxE	PfDHHC1	PFC0160w	SPG; TFxE
PbDHHC2	PBANKA_010830	DPG; TTxE	PfDHHC2	PFF0485c	DPG; TTxE
PbDHHC3	PBANKA_092730	DPL; TTxE	PfDHHC3	PF11_0217	DPL; TTxE
PbDHHC4	PBANKA_142090	DPG; TTxE	PfDHHC4	MAL7P1.68	DPG; TTxE
PbDHHC5	PBANKA_133780	NPG; TLxE	PfDHHC5	MAL13P1.126	NPG; TSxE
PbDHHC6	PBANKA_083330	NPG; TTxE	PfDHHC6	PFI1580c	NPG; -
PbDHHC7	PBANKA_124300	PPG; TTxE	PfDHHC7	PFE1415w	SPG; TTxE
PbDHHC8	PBANKA_141970	DPG; TTxE	PfDHHC8	MAL13P1.117	DPG; TTxE
PbDHHC9	PBANKA_093210	NPG; TTxE	PfDHHC9	PF11_0167	NPG; -
PbDHHC10	PBANKA_051200	SPG; TTxE	PfDHHC10	PF10_0273	NPG; TTxE
PbDHHC11	PBANKA_031260	NPG; -	PfDHHC11	PFB0725c	NPG; TTxxE
-	-	-	PfDHHC12	PFB0140w	DPG; TxxE

Table 4.1: DHHC-domain-containing proteins in *Plasmodium berghei* and *Plasmodium falciparum*. The nomenclature of the DHHC proteins in both parasite species are shown along with their *Plasmo*DB accession numbers. The two additional conserved motifs (besides the characteristic DHHC domain) are also indicated.

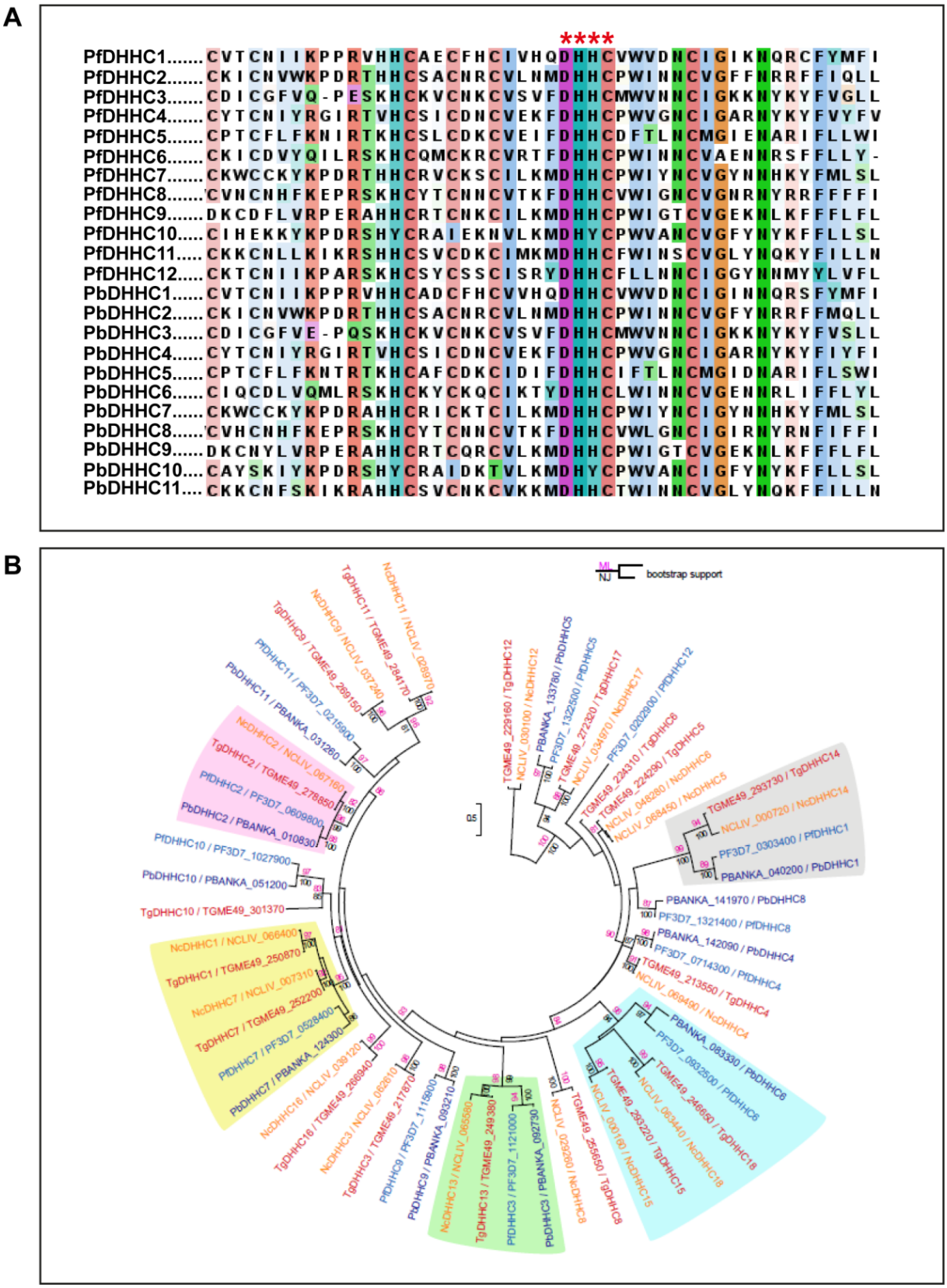


Figure 4.1: Repertoire of DHHC-containing proteins in *Plasmodium*. (A) Multiple sequence alignment of DHHC-domain containing proteins in *P. falciparum* and *P. berghei*. The amino acid sequences of the DHHC-cysteine rich domains of all the DHHC proteins in *P. falciparum* and *P. berghei* are shown aligned using ClustalW2 and Jalview. The characteristic DHHC motif is highlighted with asterisks. **(B)** Phylogenetic tree of the DHHC protein family in *P. falciparum*, *P. berghei*, *T. gondii* and *N. caninum* based on NJ distance analysis and ML analysis [4].

Advances in *P. berghei* genetic modification technology, developed by the Billker and Rayner labs, now allows quicker, large-scale and much more efficient means of genetic manipulation in *P. berghei* [5]. Thus, the study of the *Plasmodium* DHHC proteins was chosen to be performed first in *P. berghei*, where analysis of the full repertoire of DHHC proteins was feasible, before following up specific DHHC proteins of interest in *P. falciparum*. In order to analyse all the *Plasmodium* DHHC proteins in *P. berghei*, vectors from the *Plasmo*GEM resource (http://plasmogem.sanger.acuk) [5] were used to introduce a C-terminal triple-HA (3-HA) epitope tag to as many of the PbDHHC proteins as possible, as well as to perform knock-out studies on these same PbDHHC genes.

4.2. Expression and localisation of DHHC proteins in *Plasmodium berghei*

4.2.1. Generation of PbDHHC triple-HA (3-HA)-tagged transgenic parasite lines

As described above, there are 11 DHHC-containing proteins in *P. berghei,* now referred to as PbDHHC1 to 11. Tagging vectors from the *Plasmo*GEM resource [5] were available for 9 of the PbDHHC genes (PbDHHC3-11), and these vectors were introduced into *P. berghei* strain ANKA 2.34 as described in the Materials and Methods. Integration of the tagging constructs was expected to occur by double-crossover recombination of the homologous regions, resulting in the removal of the STOP codon and the insertion of the 3-HA tag, as well as the insertion of the *hDHFR/yfcu* selection cassette, which confers resistance to the drug pyrimethamine [6] (Figure 4.2A). The successful integration of each of the nine PbDHHC tagging constructs into the expected chromosomes was demonstrated by Southern blotting of chromosomes separated by pulsed field gel electrophoresis (PFGE) (Figure 4.2B), indicating the generation of 3-HA-tagged transgenic lines for 9 of the PbDHHCs (PbDHHC3 to 11).

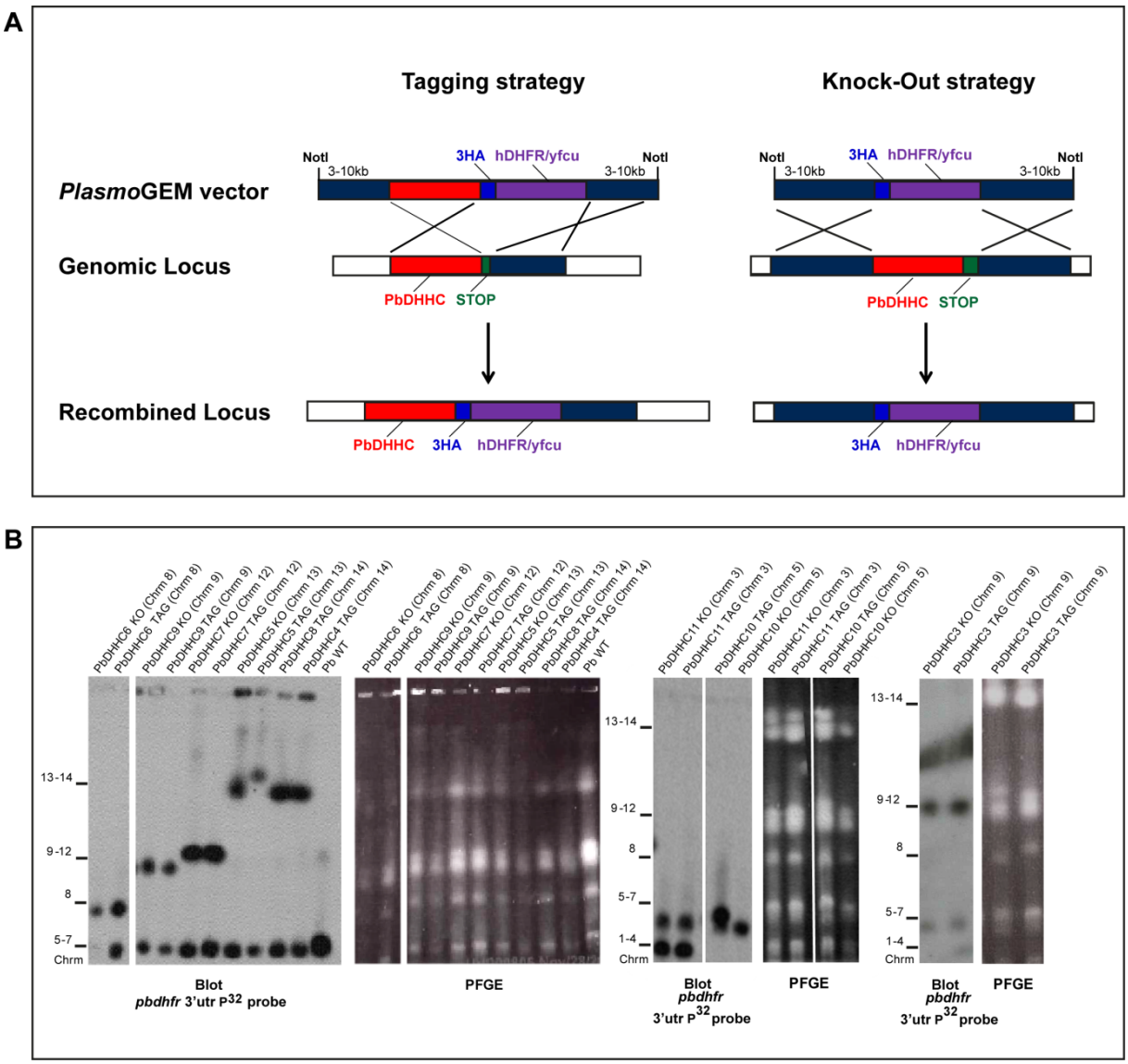


Figure 4.2: Generation of PbDHHC triple-HA-tagged and knock-out transgenic lines. (A) Scheme of the strategy used to C-terminally-tag and knock-out the endogenous locus of the PbDHHC genes of interest. **(B)** Pulsed field gel electrophoresis (PFGE) and southern blot analysis of size-separated *P. berghei* chromosomes using a probe specific to the PbDHFR 3' UTR, indicating the integration of the tagging and knock-out vectors into the expected chromosomes.

4.2.2. Five DHHC proteins are expressed in P. berghei schizont stages

In order to confirm that the 3-HA-tagged PbDHHC proteins from the transgenic parasite lines were expressed in parasite blood-stages, immunoblots of purified schizont preparations, obtained from all of the nine 3-HA-tagged transgenic *P. berghei* lines, were performed using antibodies against the 3-HA epitope tag. It is important to note that the tagging strategy results in integration of the 3-HA epitope at the endogenous locus, meaning that the PbDHHC protein is still expressed under its native promoter.

The predicted sizes of the PbDHHC proteins listed on *Plasmo*DB (http://plasmodb.org) ranges from 35 kDa to 160 kDa. As all the PbDHHC proteins have 4 TM-domains, the difference in their sizes is mainly due to the presence or absence of ankyrin repeats in their N-terminal domains, or in the case of PbDHHC4, a very long C-terminal domain. Immunoblot of the PbDHHC schizont extracts revealed single bands that ran at the expected sizes for five of the nine PbDHHC schizont preparations (PbDHHC3, 5, 7, 8 and 9) as shown in Figure 4.3.

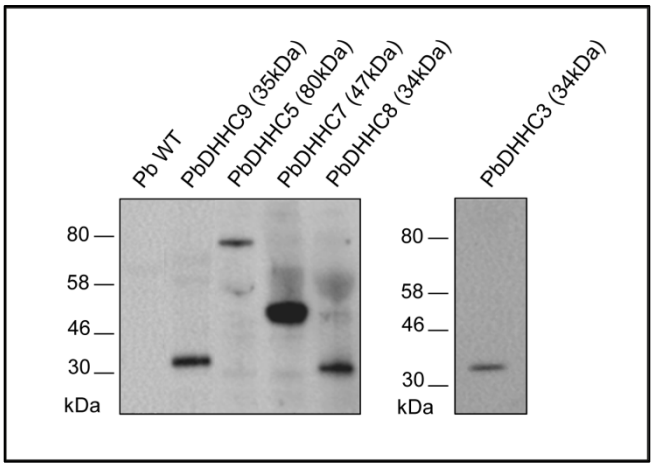


Figure 4.3: Expression of PbDHHC proteins in purified *P. berghei* schizonts. Immunoblot analysis was performed on total protein extracts from purified *P. berghei* schizonts, expressing the 3-HA-tagged PbDHHC proteins. Membranes were probed with α-HA antibodies and the expected protein sizes are shown in brackets.

Bands were not detected for the remaining tagged DHHC proteins (PbDHHC4, 6, 10 and 11). This could be due to low expression levels of the tagged protein. Alternatively, the inability to detect the protein could be due to stage-specific expression. For the purposes of this work, only the intraerythrocytic schizont stages were studied and it is possible that the DHHC proteins not detected in schizonts could perhaps be expressed at different stages, either of the intraerythrocytic cycle (for

example, ring or trophozoite stages), or of the whole life cycle (for example, the liver or mosquito stages). Some transcription data supports such an explanation for some of the DHHC genes – RNAseq data indicates that the expression of *P. falciparum* homologues of PbDHHC6 and 10 appear to be more up-regulated in gametocyte stages [7], and neither PbDHHC6 or 10 were detected in schizonts here. This suggests that while *Plasmodium* parasites have a similar number of DHHCs as other single-celled eukaryotes, such as *S. cerevisiae*, because of their complex life cycle, they may rely on a more restricted subset of these genes at specific life cycle stages.

4.2.3. Subcellular localisation of DHHC proteins in P. berghei

In other eukaryotes, DHHCs are predominantly found in the ER and the Golgi. For example, the yeast DHHC-PAT, Erf2 localises to the ER [8], while another DHHC-PAT, Akr1, localises to the Golgi [1]. However, *Plasmodium* parasites possess several unique organelles not found in other species. In order to establish the subcellular localisation of *Plasmodium* DHHCs, and specifically to determine whether any were localised to the unique parasite organelles, immunofluorescence microscopy was performed on the parasite lines containing 3-HA-tagged versions of the five PbDHHC proteins (PbDHHC3, 5, 7, 8 and 9) that could be detected by immunoblot. All five of these PbDHHCs appeared to distribute to discrete foci in *P. berghei* schizonts (Figure 4.4). The possible localisations of these proteins were determined by comparing the immunofluorescence staining of all five PbDHHC proteins to that of two proteins with well-established localisations, for which high quality antibodies were available: ERD2 (Golgi marker) [9] and merozoite surface protein 1 (MSP1) (plasma membrane marker) [10].

PbDHHC8 displayed an intracellular staining which did not co-localise to either ERD2 or MSP1 (Figure 4.4D). Further investigation using the endoplasmic reticulum (ER) marker, BIP [11], revealed that PbDHHC8 also did not co-localise with BIP (Figure 4.4D). However, this intracellular discrete staining, that was not Golgi or ER staining, could suggest localisation to vesicles. Unfortunately, the number of available localisation markers in *P. berghei* is limited, and thus, the specific location of PbDHHC8 is as yet unknown.

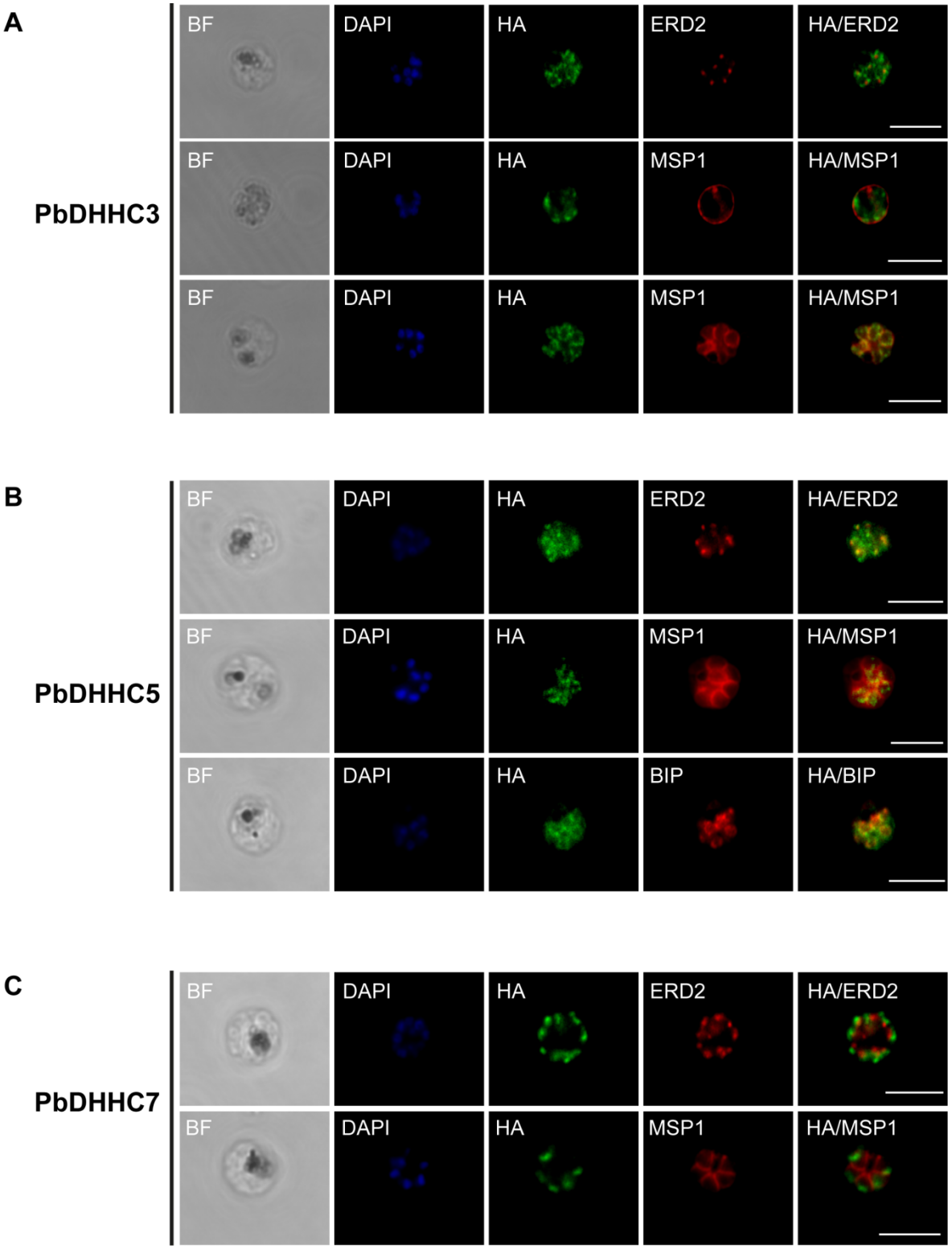
Both PbDHHC3 and PbDHHC9 appeared to co-localise with MSP1 in late schizonts (Figure 4.4A and E). However, this co-localisation was not observed in earlier stages of schizogony (data shown for PbDHHC3, Figure 4.4A), suggesting an inner membrane complex (IMC) localisation rather than a plasma membrane localisation. The IMC is a distinct morphological feature of alveolate organisms such as *Plasmodium*, and consist of flattened, membranous vesicles that underlie the plasma

membrane. In mature schizonts where the IMC is fully developed, it lies closely beneath the plasma membrane, and at this stage, staining of the IMC is closely similar to staining of the plasma membrane [12]. However, in the earlier stages of the life cycle, such as the ring and trophozoite stages, as well as during the early stages of schizogony, the IMC is still in the process of developing, and does not yet lie closely against the plasma membrane. At these stages, the staining of the IMC appears more punctate, and does not appear similar to the plasma membrane [12]. Thus, as this was observed for both PbDHHC3 and 9, it appears that these PbDHHC proteins are localised to the IMC rather than the plasma membrane.

PbDHHC5 did not co-localise with MSP1 but displayed some partial co-localisation with ERD2 (Figure 4.4B). Further investigation using the ER marker, BIP, also revealed partial co-localisation of PbDHHC5 with BiP (Figure 4.4B), suggesting both a Golgi and ER localisation.

Lastly, PbDHHC7 appeared to have a punctate distribution, different from that of ERD2 (Figure 4.4C). Co-staining with MSP1 suggested that PbDHHC7 was apically located (Figure 4.4C). This suggests possible rhoptry localisation. Unfortunately, localisation to the rhoptries was unable to be confirmed due to the lack of antibodies against rhoptry markers in *P. berghei.*

The remaining five PbDHHC proteins (PbDHHC4, 6, 10 and 11) were unable to be detected by immunofluorescence assay, again perhaps due to low expression levels or stage-specific expression.


A
PbDHHC3
BF
DAPI
HA
ERD2
HA/ERD2
BF
DAPI
HA
MSP1
HA/MSP1
BF
DAPI
HA
MSP1
HA/MSP1
B
PbDHHC5
BF
DAPI
HA
ERD2
HA/ERD2
BF
DAPI
HA
MSP1
HA/MSP1
BF
DAPI
HA
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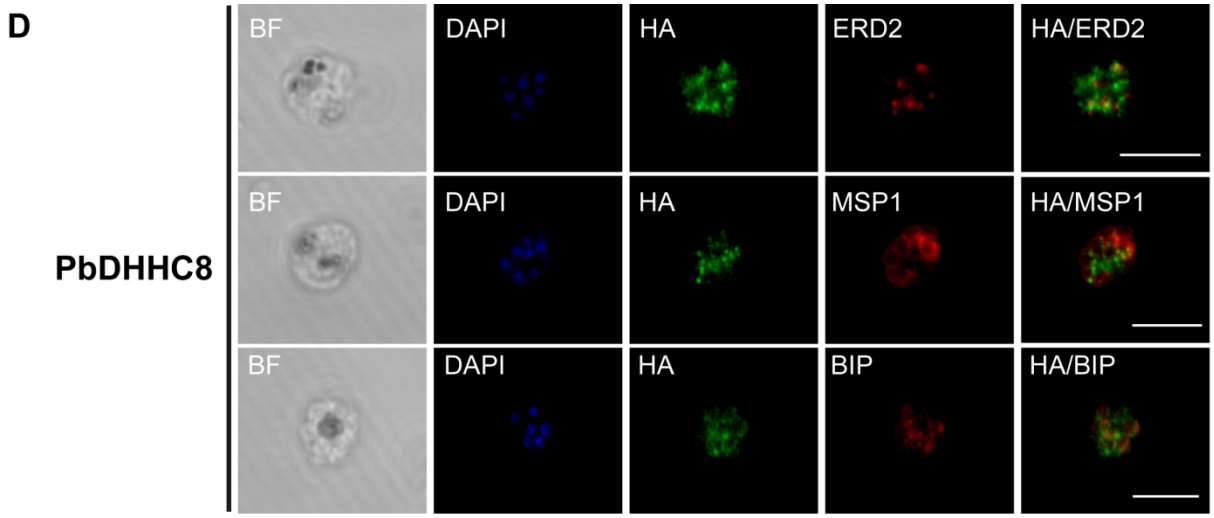
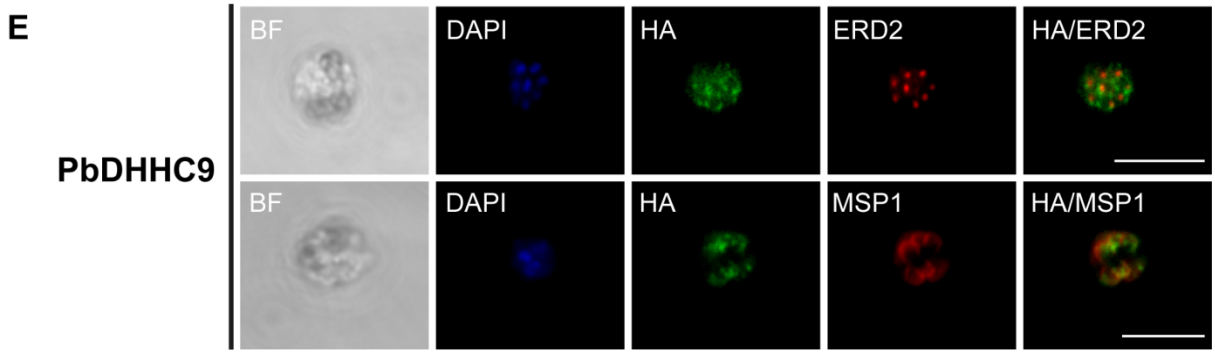
HA/ERD2
BF
DAPI
HA
MSP1
HA/MSP1


D
PbDHHC8
BF
DAPI
HA
ERD2
HA/ERD2
BF
DAPI
HA
MSP1
HA/MSP1
BF
DAPI
HA
BIP
HA/BIP


E
PbDHHC9
BF
DAPI
HA
ERD2
HA/ERD2
BF
DAPI
HA
MSP1
HA/MSP1

F

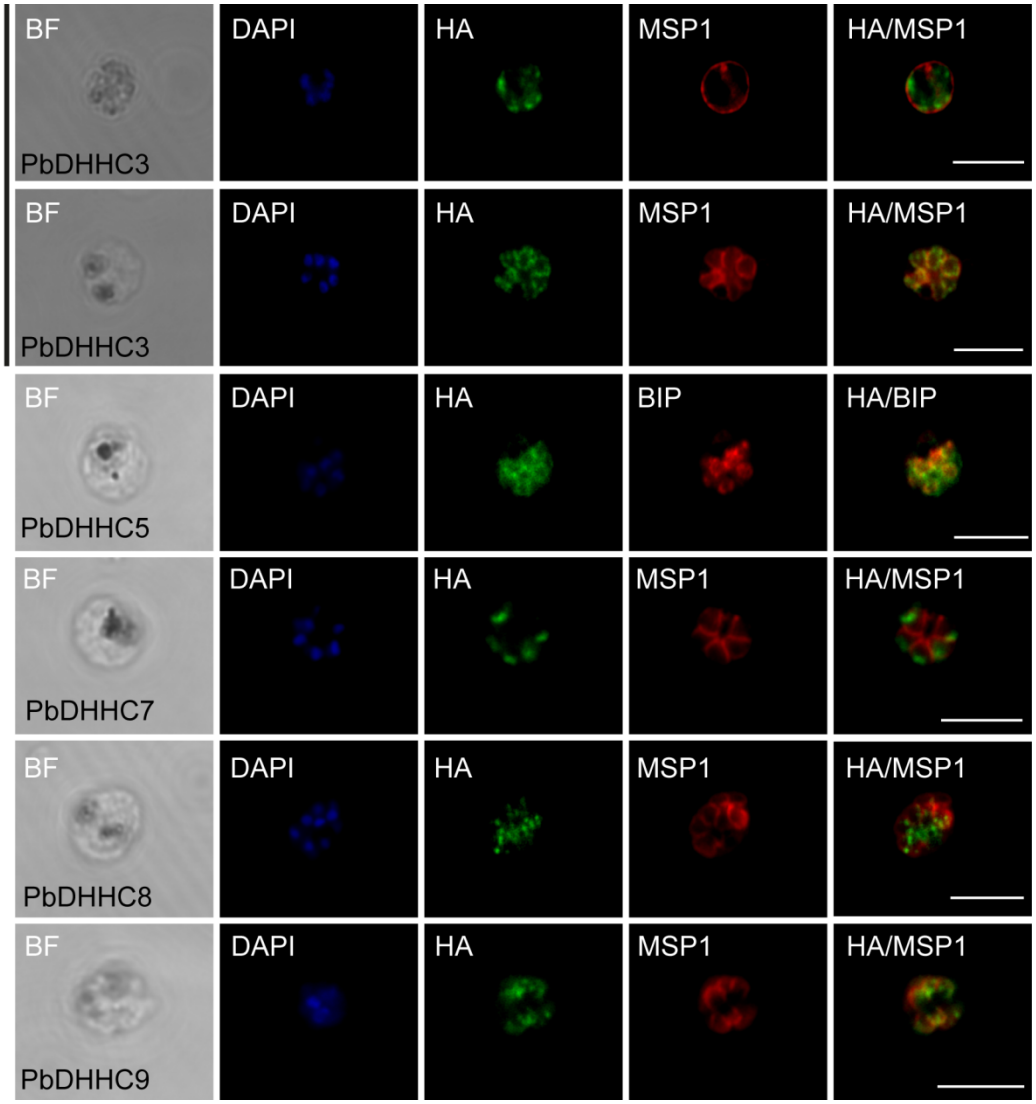


Figure 4.4: Localisation of DHHC proteins in *P. berghei* schizonts. Triple-HA-tagged PbDHHC proteins were localised by immunofluorescence using antibodies against the 3-HA tag (green) and comparing their immunofluorescence staining against that of the following known localisation markers (red): ERD2 (Golgi marker), MSP1 (plasma membrane marker), BIP (endoplasmic reticulum marker). Nuclear staining by DAPI is shown in blue. **(A)** Localisation of PbDHHC3. Staining is shown for both an early schizont and a late schizont in order to differentiate IMC from plasma membrane, as IMC staining is similar to plasma membrane staining in late stage schizonts but not early schizonts. **(B)** Localisation of PbDHHC5. **(C)** Localisation of PbDHHC7. **(D)** Localisation of PbDHHC8. **(E)** Localisation of PbDHHC9. **(F)** Summary of the localisations of the five 3-HA-tagged PbDHHC proteins. Scale bar: 5 µm.

The localisation data described above has revealed that the five PbDHHC proteins expressed in *P. berghei* schizonts appear to be localised to four different sites within the parasite. This includes typical DHHC localisations, such as the ER and Golgi, as well as parasite specific organelles, such as the IMC and potentially the rhoptries (Figure 4.4F). The fact that the DHHC proteins appear to be distributed to different locations in the parasite can imply two things. Firstly, the substrate specificity of the DHHC proteins could be governed by their location, in that the DHHC proteins only come in contact with a certain subset of target proteins at their specific location, thus regulating the specificity of the enzyme-substrate binding of these DHHC proteins. Secondly, palmitoylation of target proteins could be regulated to occur only at a specific organelle, that is, the target protein is only palmitoylated when transported to the particular organelle where its DHHC-PAT is located, which may be important in regulating protein activity.

The generation of the tagged transgenic parasite lines, PFGE analysis of the tagged lines, and isolation of infected mouse blood described in this section were performed by Ellen Bushell, Billker and Rayner lab, while I performed the *P. berghei* schizont purification, parasite protein extraction, immunoblotting, immunofluorescence assay, and the acquiring of images by confocal microscopy.

4.3. Essentiality of the DHHC proteins *in P. berghei*

Knock-out vectors from the *Plasmo*GEM resource [5] were available for 9 out of the 11 PbDHHC genes (PbDHHC3-11), and were introduced into *P. berghei* strain ANKA 2.34 as described in the Materials and Methods. Integration of the knock-out vectors was expected to occur via double-crossover recombination of the homologous regions, resulting in the removal of the PbDHHC gene of interest, and its replacement with the *hDHFR/yfcu* gene selection cassette, as shown in Figure 4.2A.

PFGE confirmed the integration of the knock-out vectors into the expected chromosomes for 7 of the 11 PbDHHC genes: PbDHHC3, 5-7 and 9-11, indicating the successful generation of transgenic knock-out lines for these seven PbDHHC proteins (Figure 4.2B). This indicates that these seven PbDHHCs are not essential for *P. berghei* asexual growth, and thus suggests that these genes could be functionally redundant in the asexual intraerythrocytic stages. Additionally, two of the PbDHHC proteins successfully knocked-out here -PbDHHC6 and 10- had no detectable blood-stage expression by epitope-tagging, as described above. This further supports the possibility that these proteins could be functionally important in another life cycle stage besides the intraerythrocytic stages.

Although knock-out vectors were available for PbDHHC4 and 8, attempts to generate transgenic knock-out lines for these genes were unsuccessful. This could be an indication that these genes are not amenable to disruption, suggesting that these genes could be essential for blood-stage growth. However, confirmation of this hypothesis, by providing an episomally-expressed gene copy whilst simultaneously disrupting the gene locus, was not within the scope of this project.

Nevertheless, the results described here suggest that a there may be two subsets of DHHC proteins in *P. berghei*: one subset appears to be functionally redundant in the blood-stages, whereas the other subset of DHHC proteins could be essential for blood-stage growth.

The generation of the knock-out transgenic lines and PFGE analysis of the knock-out lines described in this section were performed by Ellen Bushell, Billker and Rayner labs, and this data is included here due to its relevance to work described in other later chapters.

The work described in this chapter has since been published in [4].

Conclusion

This analysis of the repertoire of DHHC-containing-proteins in *P. berghei* has provided several important insights. Firstly, only a subset of DHHC proteins appears to be expressed in schizonts, implying that the other DHHC proteins not detected in schizonts could be expressed in other life cycle stages of the parasite. Additionally, the fact the some DHHC proteins appear to be essential for blood-stage growth, while other DHHC proteins seem to be redundant, further supports the suggestion that stage-specific expression exists for the *Plasmodium* DHHCs. Secondly, the PbDHHC proteins appear to have different locations within the parasite, which may have implications on how substrate specificity of the DHHCs could be regulated. However, the main focus of this project was to understand the palmitoylation site specificity of the *Plasmodium* DHHC proteins. One of the ways this could be achieved was to combine the generation of DHHC knock-out parasite lines with the site-ID palmitome purification method (described in Chapter 3), in an attempt to determine the specific cysteines palmitoylated by individual DHHC-PATs. The palmitome is more well-established in *P. falciparum,* and large amounts of material for biochemical approaches can be more easily generated using *P. falciparum.* Thus, the next step in this study was to investigate the DHHC-domain-containing proteins in *P. falciparum*, focusing on those that are more highly expressed in schizonts.

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