Chapter 7

Discussion

Palmitoylation, one of the lesser known protein PTMs, was initially thought to be merely a simple membrane anchor. Recently however, the development of new methods to purify whole 'palmitomes' from individual organisms has revealed the broad scope of this PTM in many different eukaryotic organisms, and has raised questions about the potentially diverse roles played by this PTM in protein function. The fact that palmitoylation is the only known reversible lipid modification, as well as evidence that palmitoylation can have active roles in many aspects of protein localisation, trafficking and function, has brought about the view that this protein PTM is important for normal cellular function [1, 2]. Furthermore, the recent discovery of protein families capable of mediating the addition of palmitate to proteins (the DHHC and MBOAT protein families), as well as protein families capable of removing palmitate from proteins (the APT protein family), raises interesting questions regarding the dynamic regulation of palmitoylation, and the effect and role of this dynamic regulation on the function of the palmitoylated protein [2, 3]. Until recently, only three proteins were confirmed to be palmitoylated in *Plasmodium* parasites (GAP45 [4], CDPK1 [5] and calpain [6]). Application of the new palmitome purification technologies however has revealed that more than 400 putative palmitoyl-proteins are found in the intraerythrocytic schizont stages of *P. falciparum*, which is a similar percentage of the total proteome as found in other eukaryotic species [7]. In the work described in this dissertation, a more in-depth study of protein palmitoylation in *P. falciparum* was performed, and can be divided into two main aims – a global analysis of the sites of palmitoylation within the palmitome of *P. falciparum* schizonts, and the characterisation of the DHHC and MBOAT proteins in *Plasmodium* parasites.

In order to fulfil the first aim, a method for the purification of palmitoylated peptide fragments was developed based on previously used purification techniques. This method was applied to the schizont stages of *P. falciparum* and resulted in the successful purification of putative palmitoylated peptide fragments, which contained the putative palmitoylated cysteine residues, resulting in the identification of potential sites of palmitoylation within the *P. falciparum* schizont palmitome (Chapter 3).

Although the application of this site-ID palmitome purification method resulted in the identification a number of putative palmitoylated peptides, further development is clearly needed. Two main technical issues remain to be overcome. Firstly, the quantification method that resulted in the best proteome coverage and produced the largest dataset of enriched peptides was label-free quantification. However, this method of quantification is less accurate and more prone to error and variability (due to the inherent run-to-run experimental variations which occur during label-free mass spectrometry analysis) compared to other methods of quantification, such as SILAC metabolic

labelling and stable isotope dimethyl labelling [8, 9]. This means that the datasets produced, although certainly containing actual palmitoylated peptides, may possibly contain more false-positive identifications due to inaccurate quantification, and subsequently, generation of inaccurate enrichment ratios. These datasets must therefore be treated with higher stringency when deciding the cut-off point for enrichment. One way to combat this would be to perform many replicates of different purifications and considering only the peptides which are consistently identified as enriched across the replicates, as consistent peptide identifications across multiple replicates are less likely to be false-positive contaminants. For the site-ID palmitome purifications from *P. falciparum* schizonts described here, multiple label-free replicates were unable to be performed due to time constraints. However, by considering the overlaps between the datasets produced from all the different purifications performed during the development of the purification method, and accepting only the peptides that were consistently present in the different datasets, palmitoylation sites could still be identified, which will be valuable for follow-up experiments.

The second issue observed during the development of the site-ID palmitome purification method was the relatively high background of cysteine-containing peptides identified in control samples. A 'noise' level of cysteine-containing peptides is always expected to be present in control samples, and in fact, is necessary for relative quantification of abundance by mass spectrometry. In these analyses, enrichment ratios were used as a measure of the enrichment of a particular peptide in the palmitome samples compared to the control samples, and thus as an indication of whether the peptide was palmitoylated or not. These enrichment ratios were generated by comparing the intensity of the particular cysteine-containing peptide in palmitome samples with its intensity in the control samples. This means that a level of cysteine-containing peptides in control samples is required for the generation of the enrichment ratios. The abundance of these cysteine-containing peptides in control samples are usually very low and are not usually high enough to be directly sequenced and detected by MaxQuant. These peptides are instead usually found by MaxQuant in the control samples using the 'match between runs' feature, which matches peptide ions by their mass and retention time. However, in these site-ID purifications, some cysteine-containing peptides in control samples was directly identified by MaxQuant, indicating that the background of cysteine-containing-peptides in the control samples was unfortunately higher than expected.

This high background in control samples would not have a great effect on peptides which had much higher intensities in the palmitome samples, thus resulting in high enrichment ratios. However, this background could certainly affect those peptides with lower intensities, and could possibly in fact mask the fold enrichment of the peptide. This is further complicated by the fact that label-free

quantification is less sensitive to small quantitative differences [9], and thus might not be able to accurately measure the lower fold enrichment due to increased background in the control samples. This background of cysteine-containing peptides could thus have an effect on the enrichment ratios, and this would ultimately affect the classification of peptides as enriched, as the cut-off criteria used here to define enrichment is based on the enrichment ratios (described in Section 3.1.2). This is especially true when the cut-off criteria is based on the median of the enrichment ratios, as the generation of relatively low enrichment ratios as a result of the background in the control would result in a lower median, and subsequently, a lowered cut-off point.

The presence of this high background of cysteine-containing peptides in the control samples points to either the incomplete blockage of free thiols by the initial IAA (or NEM) treatment, or the possibility that palmitate groups were somehow falling off during the later steps of purification. Treatment with both IAA and NEM is sensitive to pH, with specific blockage of free thiols taking place at a specific pH. The specificity of the blockage and the rate of the reaction are thus dependent on the pH of the reaction. Although the pH was measured and adjusted to the optimum pH during the initial blocking of free thiols, minor changes in the pH could have occurred during the course of the blockage step (the samples were usually blocked for two hours), especially since a reducing agent, TCEP, was present in the lysis buffer for the extraction of the proteome, in order to reduce any disulphide bonds present. This could potentially contribute to the background observed in control samples, although it is unlikely to be the only cause of the high background in the control. Unfortunately, due to time constraints, this issue was unable to be solved before the writing of this dissertation.

However, despite the presence of this background in the datasets already obtained, analysis of the overlaps between the datasets, as described above, can still provide useful information on the potential sites of palmitoylation. By considering just the peptides that were consistently purified in the two label-free datasets (Trial 3B and 4), and which corresponded to proteins previously identified in the total schizont palmitome [7], 142 putative palmitoylation sites were identified. Furthermore, of these 142 common enriched palmitoylation sites, 14 palmitoylation sites were found to be common across four of the trial purification datasets (Trial 2, 3A, 3B and 4). Although false positive identifications are undoubtedly still present within these datasets, not only due to the issues reported above, but also due to the contaminant proteins that are inherently purified by ABE methods [7], this list of potential palmitoylation sites can still be used as a guide for the design of further experiments and the generation of testable hypotheses.

It was interesting to note that in all the datasets collected, not only the *P. falciparum* schizont dataset, but also the preliminary *P. berghei* schizont dataset, as well as the *T. gondii* tachyzoite dataset, members of the DHHC protein family were found to be present and were classified as enriched. The DHHC protein family have been shown to be the enzymes responsible for catalysing protein palmitoylation in other eukaryotic organisms, and all the DHHC proteins studied thus far have been shown to also be palmitoylated themselves [3]. The identification of these proteins in the site-ID palmitome purification datasets is therefore reassuring, not only as additional validation for the site-ID palmitome purification method, but also as possible confirmation that these proteins are also palmitoylated in Apicomplexan organisms. Each of the three Apicomplexan species mentioned above possess a relatively large repertoire of DHHC-domain-containing proteins, with 12 DHHC proteins in *P. falciparum*, 11 DHHC proteins in *P. berghei* and 18 DHHC proteins in *T. gondii.* However, not all of the DHHC proteins were identified in the different site-ID trial purifications, and in fact only one of the PfDHHC proteins was consistently identified in more than one of the *P. falciparum* trial purifications. This may yet again be the result of the issues faced with the site-ID protocol (as described above), or may instead reflect the difficulties of sufficiently extracting these multi-pass TM-domain-containing proteins, some of which have relatively high molecular weights. Nevertheless, the identification of some members of the DHHC protein family in these site-ID purification datasets was a first step in the characterisation of this protein family in Apicomplexan parasites.

Importantly, in all the site-ID trial datasets, the cysteine residue identified as palmitoylated for the DHHC proteins was not the cysteine residue of the conserved DHHC motif. One hypothesis for the transfer of palmitate to cysteine residues of target proteins involves the formation of a palmitoyl-enzyme intermediate, and the cysteine of the DHHC motif has long been thought to be a possible candidate for the site of modification on DHHC proteins, although modification at another cysteine residue is also thought to be possible [3]. The data obtained from the site-ID palmitome purifications however indicate that palmitoylation of the DHHC proteins appeared to occur on a cysteine residue which was not the cysteine of the DHHC motif, at least for the DHHC proteins identified in the site-ID purifications. It is possible that the cysteine of the DHHC motif is also palmitoylated but was not identified in the site-ID purifications, due either to chance or to the issues with the site-ID protocol mentioned above, and the cysteine identified as palmitoylated by the site-ID purifications could perhaps have other roles besides palmitate transfer, such as in the localisation of the DHHC protein itself or in regulating substrate specificity. Conversely, another possibility is that the cysteine residue involved in palmitate transfer might not be the cysteine of the DHHC motif, if palmitate transfer does indeed occur via a palmitoyl-enzyme intermediate. Whether these cysteine residues are truly palmitoylated, and whether palmitoylation at these sites are actually required for the palmitoylation

of the target proteins by the DHHCs can now be validated and tested using the PAT activity assay also developed in the work for this dissertation (Chapter 6), and this may then provide some insight into the mechanisms of palmitate transfer by the DHHC proteins.

In order to further characterise these putative PATs in *Plasmodium* parasites, the repertoire of DHHC-domain-containing proteins were localised and knocked-out in both species of *Plasmodium*: *P. berghei* and *P. falciparum* (Chapter 4 and 5). In both *Plasmodium* species, the DHHC proteins appeared to localise to different membrane-bound compartments within the cell. These membrane compartments consisted of organelles such as the Golgi [10] and the ER [11], to which the DHHC proteins of other eukaryotic organisms are commonly localised to, as well as specialised, parasite-specific organelles, such as the rhoptries and the IMC. The distribution of the DHHC proteins to various different membranous compartments suggests that at least one aspect of the regulation of DHHC protein substrate specificity may be reliant on the localisation of the DHHC protein, which dictates the potential target proteins that come into contact with the particular DHHC protein. Additionally, the expression of the DHHC proteins appeared to be stage-specific, with only a subset of proteins highly expressed in the schizont stages studied here, which further implied that the activity of different DHHC proteins may be required at different stages of the parasite life cycle. The localisation of these proteins to different sites in the cell, and the differential expression of these proteins at different stages of the life cycle, may be the reason why so many of the DHHC proteins are present in each species, and may be how substrate specificity of these proteins is regulated, especially considering the fact that these proteins have been found to be relatively promiscuous in terms of substrate specificity in other organisms [12].

Studies on the essentiality of the DHHC protein family in both *Plasmodium* species revealed the existence of two subsets of DHHC proteins. One subset consisted of proteins that were unable to be knocked-out in the intraerythrocytic stages of the parasite, implying that these proteins were essential for blood stage growth, and the second subset consisted of proteins that were successfully knocked-out in the blood stages without any detrimental effect on blood stage growth, implying a possible redundancy in the function of these proteins, at least in the blood stages studied here. Overlapping functionality of the DHHC proteins has been demonstrated before in yeast, where individual deletions of yeast DHHC proteins had minimal effect on substrate palmitoylation. In fact, a significant decrease in substrate palmitoylation was only observed when multiple DHHC proteins were simultaneously deleted [12]. The ability to delete some of the DHHC proteins in both *P. berghei* and *P. falciparum* imply that the same overlapping functionality may exist in *Plasmodium.* However, whereas all the yeast DHHC proteins were found to be non-essential when individually deleted [12],

the results shown here indicate that at least a few of the DHHC proteins in *P. falciparum*, for example PfDHHC3 (localised to the Golgi), PfDHHC7 (localised to the rhoptries) and PfDHHC8 (localisation undetermined due to low expression), and in *P.* berghei, for example, PbDHHC4 (localisation unknown) and PbDHHC8 (localisation undetermined due to low expression), appeared to be essential to blood stage growth, perhaps reflecting the more complicated life cycle of the parasite and possible life cycle stage-specific expression and function of the DHHC proteins.

Due to time constraints, only the intraerythrocytic stages were studied here for both *P. falciparum* and *P. berghei*. Recent work by others however, revealed that the disruption of PfDHHC9 (which was shown in the work presented in this dissertation to be non-essential in the blood-stages of both *P. falciparum* and *P. berghei*) prevented the formation of mature gametocytes in *P. falciparum*, implying that this DHHC protein is essential for gametocytogenesis [13], a process which is necessary for the transmission of the parasite to the mosquito vector. This indicates that further work on the localisation and essentiality of the DHHC proteins in other stages of the parasite life cycle besides the asexual intraerythrocytic stages, such as the sexual gametocyte stages, would certainly provide further insight on the function and importance of the DHHC protein family in *Plasmodium* biology. These studies could be more easily done using *P. berghei* instead of *P. falciparum*, as the phenotype of a particular DHHC knock-out strain could be observed in all stages of the *Plasmodium* life-cycle, and the effect of the disruption of the DHHC protein on parasite transmission to the mosquito could also be studied.

The next step in understanding the specificity of palmitoylation in *Plasmodium* parasites would be to combine the DHHC knock-out transgenic parasite lines generated here with the newly-developed site-ID palmitome purification protocol, in order to determine which specific or overlapping sites were palmitoylated by individual DHHCs. This would be achieved by performing the site-ID palmitome purification on each individual DHHC knock-out transgenic strain, and comparing the enriched palmitoylation sites obtained, with that of the wild-type strain, in order to determine whether any palmitoylation sites were no longer present when a particular DHHC protein was deleted. This would then be an indication of which sites were palmitoylated by a specific DHHC protein. Similar studies in yeast revealed that the yeast DHHC proteins palmitoylate overlapping sets of substrate proteins [12]. However, given that unlike in yeast, some of the DHHC proteins in *Plasmodium* are not redundant and are not amenable to disruption in the blood stages, this might not be the case for this set of non-redundant DHHC proteins, which may possibly be responsible for palmitoylating specific, non-overlapping substrates. In order to perform the comparative site-ID studies on these essential DHHCs however, conditionally regulated versions of the essential DHHCs

would be required. While there are currently no robust regulatable systems in *Plasmodium*, recent developments, such as the DiCre conditional recombinase system [14] may offer the way forward.

Given the issues with the site-ID palmitome purification method (described above), this sort of comparative palmitoylation site analysis may potentially be difficult and complex. However, if a sufficient number of replicates are performed, and stringent and rigorous analysis applied to the datasets, valuable information can still be gleaned. Also, as mentioned above, label-free analysis is known to be less sensitive to small quantitative differences [9], and in the case of a comparative palmitoylation site analysis, might not be sensitive enough to detect any small changes in enrichment for a particular palmitoylation site. Thus, although the results presented in this work has determined that label-free quantitative analysis provided better coverage and larger datasets, in the case of the comparative site analysis, it might be more beneficial to use a chemical or metabolic labelling approach rather than a label-free approach, for the sake of higher quantitative accuracy. As stable isotope dimethyl labelling methods appeared to be provide the least information, SILAC metabolic labelling could be used instead, at least in the case of the *P. falciparum* knock-out strains, as this form of labelling worked well in the purification of the total schizont palmitome [7], and can be used for the efficient metabolic labelling of *P. falciparum* parasites in culture. Again due to time constraints, these comparative site analyses have not yet been performed, but are certainly the logical next steps in future experiments.

The repertoire of DHHC proteins in *Plasmodium* is relatively large, and the differences in the localisation and essentiality of these proteins suggested that these proteins may have important roles in parasite biology. Nevertheless, whether the role of these proteins in *Plasmodium* involved catalysing protein palmitoylation was still unknown. The development of the PAT activity assay in HEK293 cells described here however, revealed that the *Plasmodium* DHHC proteins did indeed exhibit PAT activity, at least for the four *P. falciparum* DHHC proteins studied (Chapter 6), indicating that the function of these proteins is indeed to mediate protein palmitoylation. Moreover, analysis using the PAT activity assay revealed that different PfDHHC proteins were able to palmitoylate the same target protein. This acted as further confirmation that, like in yeast, *Plasmodium* DHHC proteins may also possess overlapping functionalities.

Initial PAT activity experiments involving the mutation of the cysteine residue in the highly conserved DHHC domain suggested that for the *Plasmodium* DHHC proteins, the cysteine residue might not be the residue involved in palmitate transfer to target proteins. This contrasted with experiments performed in yeast, where mutation of the cysteine residue in the DHHC domain abolished the palmitoylation of substrate proteins [10, 11], but was consistent with the fact that the cysteine

residue identified as palmitoylated in some PfDHHC proteins by the site-ID palmitome purification method was not the cysteine of the DHHC motif, raising the question as to whether the transfer of the palmitoyl group to the cysteine residues of target proteins could occur via different mechanisms in *Plasmodium*. Further analysis using site-directed mutagenesis and the PAT activity assay, in order to determine which residues are responsible for palmitate transfer, may provide valuable insights into the mechanism of palmitate transfer by the *Plasmodium* DHHC-PATs, and as noted above, can be used to test the function of the palmitoylation sites identified within the DHHCs themselves. The PAT activity assay developed here can thus be used not only for the demonstration of PAT activity, but also for determining substrate specificity, and for exploring the mechanisms of palmitate transfer.

In order to perform the PAT activity assay, one of the first steps was to choose target proteins that could potentially be palmitoylated by the DHHC proteins of interest. This was done by considering both the data obtained from the site-ID palmitome purifications, as well as the localisation data of the DHHC proteins being studied. The target proteins were chosen based on evidence of palmitoylation in the site-ID datasets and their localisation in relation to the localisation of the DHHC proteins. For example, one of the target proteins chosen, PfSec22, was identified in the Trial 4 site-ID palmitome purification dataset, with a high enrichment ratio that passed the cut-off criteria for highly enriched proteins. Additionally, PfSec22 was annotated as a SNARE protein, found to be localised to the ER, which made it a candidate for palmitoylation by the DHHC proteins localised to the ER and Golgi. Subsequent experiments using the PAT activity assay revealed that PfSec22 was indeed palmitoylated, providing further validation for the ability of the site-ID palmitome purification method to truly purify palmitoylated proteins. Furthermore, it was shown that PfSec22 appeared to be mainly palmitoylated by the PfDHHC proteins located at the ER (PfDHHC5) or Golgi (PfDHHC3), contributing to the possibility that substrate specificity may be affected by the localisation of the DHHC proteins in relation to the target proteins. The cysteine residues identified as potentially palmitoylated by the site-ID purification can now be validated experimentally using the PAT activity assay, in order to confirm the site of palmitoylation for PfSec22.

It also must be noted that PfSec22 was identified and classified as enriched only in the Trial 4 site-ID purification, and not in the other trial purifications. This indicates that there is the possibility that a protein which was identified in one dataset, but not in other datasets, could actually still be palmitoylated, despite the lack of overlap between datasets. Thus, when using the site-ID datasets to decide whether a protein may be palmitoylated or not, besides the overlap between the different datasets, it is also important to consider the enrichment ratio. PfSec22 for example, had a relatively

high enrichment ratio which was consistent across the two biological and two technical replicates that were performed in the Trial 4 site-ID purification. Additionally, due to the lack of overlap between datasets for a particular protein, experimental validation of palmitoylation should also be performed, which was done here in the case of PfSec22, using the PAT activity assay.

The second target chosen for the PAT activity assay was PfARO. PfARO was chosen mainly due to its localisation to the rhoptries, which made it a good candidate for palmitoylation by either PfDHHC7, which also localised at the rhoptries, or PfDHHC3 and 5 located at the Golgi and ER respectively. Additionally, PfARO was shown experimentally to be a palmitoylated protein [15], although PfARO was not identified in any of the site-ID trial palmitome purifications performed here. This thus presents a contrasting example of how these site-ID palmitome purifications can also possibly miss some truly palmitoylated proteins, perhaps due to the various issues described previously or perhaps merely by chance. Thus, although the list of putative palmitoyl-proteins and their potential palmitoylation sites is extensive, some palmitoylated proteins still may not be captured by this method of purification. Nevertheless, the datasets generated by the site-ID purifications can still act as an initial guide when looking for potential palmitoyl-proteins, and with the development of the PAT activity assay, there is now the means to easily validate the 'palmitoylation status' of these proteins without having to produce transgenic parasite strains, which particularly in *P. falciparum*, is more difficult and more time consuming.

Conclusions

In the work described here, potential palmitoylation sites were identified for a range of putative palmitoyl-proteins through the development of a site-identification palmitome purification method coupled with quantitative mass spectrometry approaches. The DHHC protein family was characterised in *Plasmodium* parasites, and found to be distributed in different cellular compartments, raising the question as to whether the localisation of the DHHC protein may play a part in regulating substrate specificity. The DHHC family was also found to consist of genes that were both essential and non-essential for blood stage growth, implying a functional redundancy for some of these DHHC proteins. It would be interesting to determine whether any of these DHHC proteins are essential for other stages of parasite development, which would imply a specific functional requirement for that DHHC protein at a particular stage of development.

Lastly, the development of the PAT activity assay allowed the PAT activity of the *Plasmodium* DHHC proteins to be demonstrated, showing for the first time that these parasite proteins did indeed act as

PATs. Studies using this PAT activity assay also confirmed that *Plasmodium* DHHC proteins exhibited overlapping functionalities and were capable of palmitoylating the same substrate, although for some target proteins, there appeared to be a preference for particular DHHC proteins. This assay can now be used to validate the palmitoylation sites identified by the site-ID palmitome purifications, as well as to try and tease out the mechanisms involved in palmitate transfer by the DHHC proteins.

Given the wide-spread occurrence of palmitoylation in *Plasmodium*, the reversibility of this lipid PTM, the presence of *Plasmodium* proteins possessing palmitoyl transferase activity, and the negative effect of the inhibition of palmitoylation on parasite growth and invasion, further pursuing the role of palmitoylation in *Plasmodium* parasites may provide novel drug targets that could be used for therapeutic interventions, especially considering the need for new therapies in the fight against malaria. For example, the set of DHHC proteins which were found to be essential for *Plasmodium* blood-stage growth are logically potential targets for future drug development. The PAT activity assay developed here could then also potentially be used to screen for compounds which inhibit the PAT activity of the essential *Plasmodium* DHHC proteins. Furthermore, the fact that one of the DHHC proteins appears to be essential for gametocytogenesis [13] raises the question as to whether this DHHC protein could be a potential transmission blocking target, and whether there are other DHHC proteins which act at similar stages of the life cycle and could thus also be used as transmission blocking drug targets. However, it must be noted that DHHC proteins are also present in humans (there are 23 DHHC proteins in humans), and the human DHHCs play essential roles in normal cellular function [16, 17]. Targeting the *Plasmodium* DHHC proteins could possibly also result in the cross-inhibition of the human DHHC proteins. An ideal drug would thus be one which can specifically target the *Plasmodium* DHHC proteins without affecting the DHHC proteins and palmitoylation in the human host. Clearly, a better understanding of palmitoylation and the DHHC proteins in *Plasmodium* parasites is still required before this can be successfully achieved. Nevertheless, the work described in this dissertation has provided new information about this lesser known protein modification in *Plasmodium*, and also provided useful assays that can hopefully contribute to achieving this goal.

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