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Extensive lysine acetylation occurs in evolutionarily conserved metabolic pathways and parasite-specific functions during *Plasmodium falciparum* intraerythrocytic development

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Summary

Lysine acetylation has emerged as a major post-translational modification involved in diverse cellular functions. Using a combination of immunoisolation and liquid chromatography coupled to accurate mass spectrometry, we determined the first acetylome of the human malaria parasite *Plasmodium falciparum* during its active proliferation in erythrocytes with 421 acetylation sites identified in 230 proteins. Lysine-acetylated proteins are distributed in the nucleus, cytoplasm, mitochondrion and apicoplast. Whereas occurrence of lysine acetylation in a similarly wide range of cellular functions suggests conservation of lysine acetylation through evolution, the *Plasmodium* acetylome also revealed significant divergence from those of other eukaryotes and even the closely related parasite *Toxoplasma*. This divergence is reflected in the acetylation of a large number of *Plasmodium*-specific proteins and different acetylation sites in evolutionarily conserved acetylated proteins. A prominent example is the abundant acetylation of proteins in the glycolysis pathway but relatively deficient acetylation of enzymes in the citrate cycle. Using specific transgenic lines and inhibitors, we determined that the acetyltransferase PfMYST and lysine deacetylases play important roles in regulating the dynamics of cytoplasmic protein acetylation. The *Plasmodium* acetylome provides an exciting start point for further exploration of functions of acetylation in the biology of malaria parasites.

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Introduction

With ~ 250 million clinical cases and a death toll of ~ 0.9 million per year, malaria remains a significant public health problem in many tropical and subtropical countries. Of the four human malaria parasites, *Plasmodium falciparum* causes the most severe form of disease and the majority of malaria-associated mortality. Whereas recent reduction in global malaria incidence has inspired renewed hopes for malaria elimination and eradication, the malaria control campaign still encounters many challenges. In particular, the parasite is notorious for developing resistance to most currently used antimalarial drugs. Therefore, continued research towards the development of novel diagnostics and therapeutics for malaria is needed, and these efforts require a comprehensive understanding of the parasite's biology.

Asexual replication of the parasites in red blood cells (RBCs) contributes to malaria-associated morbidity and mortality. Extensive microarray and proteomic analyses have established that the intraerythrocytic developmental cycle (IDC) is governed by highly regulated transcription and translation programmes (Le Roch *et al.*, 2003; Luah *et al.*, 2010; Foth *et al.*, 2011). The importance of chromatin-mediated epigenetic regulation of gene expression has been increasingly appreciated, and its roles entail many aspects of parasite biology such as cell cycle regulation, invasion and virulence (Merrick and Duraisingh, 2010; Miao *et al.*, 2010). Correspondingly, the parasite genome encodes a large suite of chromatin-remodelling and modification enzymes, among which there are at least four lysine acetyltransferases (KATs) and three classes

of lysine or histone deacetylases (KDACs or HDACs) (Horrocks *et al*., 2009; Miao *et al*., 2010). KATs catalyse the transfer of the acetyl moiety from acetyl-CoA to the ε-position of a lysine residue, whereas KDACs catalyse the removal of the acetyl group from an acetylated lysine. The reversible protein lysine acetylation is a highly regulated post-translational modification found in both prokaryotes and eukaryotes. Since the discovery of protein lysine acetylation almost five decades ago (Allfrey *et al*., 1964), studies of this modification have focused primarily on histones, the building units of nucleosomes. We and others have identified a multitude of covalent modifications on histones of *P. falciparum*, including both N-terminal acetylation and lysine acetylation (Miao *et al*., 2006; Salcedo-Amaya *et al*., 2009). Histone lysine acetylation, together with other post-translational modifications such as methylation, phosphorylation, ubiquitylation and sumoylation, profoundly affects chromatin structure and gene expression (Verdone *et al*., 2005; Shahbazian and Grunstein, 2007).

In addition to histone lysine acetylation, lysine acetylation occurs in cytoplasmic proteins. Recently, advancement in mass spectrometry (MS) allowed characterization of the 'acetylomes' in bacteria (Yu *et al*., 2008; Zhang *et al*., 2009; Wang *et al*., 2010), yeast (Henriksen *et al*., 2012), the protozoan parasite *Toxoplasma gondii* (Jeffers and Sullivan, 2012), plants (Finkemeier *et al*., 2011; Wu *et al*., 2011), *Drosophila melanogaster* (Weinert *et al*., 2011), rat (Lundby *et al*., 2012) and human cells (Kim *et al*., 2006; Choudhary *et al*., 2009; Zhao *et al*., 2010). From these studies, lysine acetylation has emerged as a widespread post-translational modification that may rival protein phosphorylation (Maurer-Stroh *et al*., 2003). Proteins with acetylated lysines participate in diverse biological functions. Particularly, lysine acetylation is highly prevalent in enzymes catalysing intermediate metabolism in both bacteria and human cells (Kim *et al*., 2006; Choudhary *et al*., 2009; Wang *et al*., 2010; Zhao *et al*., 2010). To date, studies on lysine acetylation of non-histone proteins in malaria parasites are very limited. Yet, the fact that some of the *Plasmodium* KATs such as PfMYST are localized in both nucleus and cytoplasm suggests that regulated protein lysine acetylation occurs in both compartments of the parasite (Miao *et al*., 2010). In this study, we performed a proteome-wide analysis of the *P. falciparum* acetylome during the IDC by immunoprecipitation (IP) with specific anti-acetyllysine antibodies and accurate MS. This screen identified 230 lysine-acetylated proteins belonging to considerably diverse functional groups, suggesting that acetylation plays important roles in regulating many cellular processes in *Plasmodium*. Understanding the mechanism of acetylation and its role in regulating protein functions may open a new venue for development of drugs and vaccines.

Results

Detection of lysine-acetylated proteins

To evaluate overall protein acetylation during the IDC of *P. falciparum*, Western analysis was performed with protein extracts from parasites at four time points during the IDC with anti-acetyllysine antibodies. Multiple protein bands spanning a wide mass range were detected (Fig. 1A). Several protein bands were strongly reactive to the antibodies. Competition with acetylated BSA confirmed that the protein bands detected were specific. This result also revealed that protein acetylation patterns were similar among different stages of the IDC, and the level of acetylation was the highest in late trophozoite stage, which is correlated with the most elevated haemoglobin digestion and protein synthesis in trophozoites (Fig. 1A).

KATs and HDACs in protein acetylation

Of the four known KATs in *P. falciparum*, PfMYST was found localized in both cytoplasm and nucleus, suggesting a role in acetylation of cytoplasmic proteins (Miao *et al*., 2010). To determine the involvement of PfMYST in cytoplasmic protein acetylation, we compared the overall cytoplasmic protein acetylation among trophozoites of three parasite lines, GFP-control, F1-GFP (overexpressing a full-length active version of PfMYST) and F1C3-GFP (overexpressing a truncated inactive version of PfMYST) (Miao *et al*., 2010). Overexpression of active PfMYST dramatically increased the overall acetylation levels of the cytoplasmic proteins, especially in the molecular mass range of 26–95 kDa (Fig. 1B). In contrast, overexpression of the inactive PfMYST reduced the overall cytoplasmic protein acetylation levels. This result indicated that PfMYST played a major role in the acetylation of cytoplasmic proteins (Miao *et al*., 2010). To evaluate the effect of HDAC inhibitors on protein acetylation, we treated the parasites with trichostatin A (TSA) and nicotinamide (Fig. 1C). The *P. falciparum* genome encodes five HADCs (one class I, two class II and two class III). TSA inhibits class I and II HADCs, whereas nicotinamide inhibits class III HDACs, the sirtuins (Prusty *et al*., 2008; Patel *et al*., 2009). 3D7 parasites incubated with TSA at either $1\times$ or $2\times IC_{50}$ (Patel *et al*., 2009) for 15 h did not cause significant changes in the overall acetylation patterns of cytoplasmic proteins. However, at higher TSA concentrations ($3\times$ or $4\times IC_{50}$), much higher levels of acetylation were detected (Fig. 1C). Similarly, incubation of parasites with 5, 10 or 15 mM of nicotinamide (Prusty *et al*., 2008) resulted in increases of the overall acetylation, suggesting that both class I and II HDAC and the Sir2 proteins participate in the deacetylation of cytoplasmic proteins (Fig. 1C).

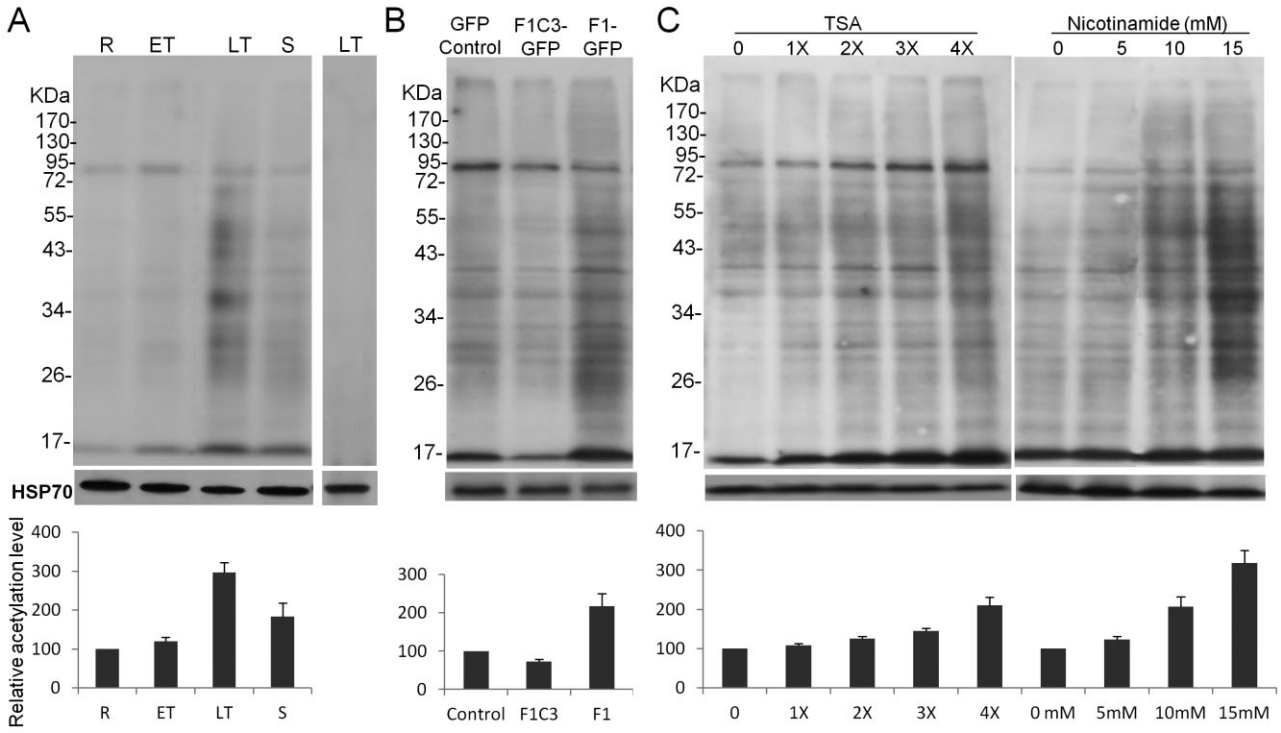

Fig. 1. Analysis of acetylation in *P. falciparum*.
A. Overall acetylation of *P. falciparum* in the intraerythrocytic development stages. Proteins were isolated from ring (R), early trophozoite (ET), late trophozoite (LT) and schizont (S). The right panel shows the reaction of anti-acetyllysine antibodies to LT proteins blocked by acetylated BSA.
B. Acetylation of cytoplasmic proteins in trophozoites of GFP-control and parasite lines with overexpression of a truncated inactive KAT PfMYST (F1C3-GFP) and overexpression of the full-length active PfMYST (F1-GFP).
C. Effect of HDAC inhibitors (TSA and nicotinamide) on cytoplasmic protein acetylation. Parasites were treated from ring stage with one to four times of IC_{50} of TSA or 5–15 mM of nicotinamide for 15 h. Equal amounts of protein lysates were separated by SDS-PAGE, and the acetylated proteins were detected with anti-acetyllysine antibodies. Equal protein loading was evidenced by Western blotting with anti-HSP70 antibodies. The graphs under the respective Western blots show the relative signal intensities determined by densitometry (mean + standard deviation) from three replicates.

P. falciparum *acetylome and distribution in cellular compartments*

To identify the *P. falciparum* acetylome during IDC, we used a similar procedure as described before (Kim *et al*., 2006; Choudhary *et al*., 2009), which integrates immunoisolation with accurate MS. Proteins from both nuclear and cytoplasmic fractions of late trophozoite stage were digested by endoproteinase Lys-C and trypsin and the resulting peptides were affinity-purified with immobilized anti-acetyllysine antibodies. The enriched peptides were analysed by 2D-LC/MS/MS and the obtained MS/MS spectra were used to search the *P. falciparum* databases with the MASCOT search algorithm (Fig. 2A). This search has essentially filtered out peptides from human protein contamination. To ensure the accuracy of the search, positive identifications were verified by manual inspections of the MS/MS spectra (the raw data can be found at http://ento.psu.edu/research/labs/liwang-cui). Since very few proteins except histones were previously reported to be acetylated in *P. falciparum* (Miao *et al*., 2006; Salcedo-Amaya *et al*., 2009; Leiva *et al*., 2012), this survey greatly expanded the inventory of lysine-acetylated proteins in this parasite with 421 lysine acetylation sites identified in 230 proteins. Although the acetylome is by no means exhaustive, the identification of gametocyte-specific proteins (e.g. pfg27) in the asexual acetylome, which might be due to very minor gametocyte contamination, suggests in-depth coverage of the acetylome. 291 and 183 lysine acetylation sites were identified in 167 and 96 proteins from the cytosolic and nuclear fractions respectively (Fig. 2B, Table S1). Whereas the two fractions shared 33 lysine-acetylated proteins, the acetylation sites were not entirely conserved, with 25 of them having different acetylation sites. Further classification of subcellular distribution of the acetylated proteins by their cellular compartment annotations was performed for the cytosol acetylome. Particular attention was paid to the mitochondrion, an organelle derived from an endosymbiotic α-proteobacterium, where widespread protein

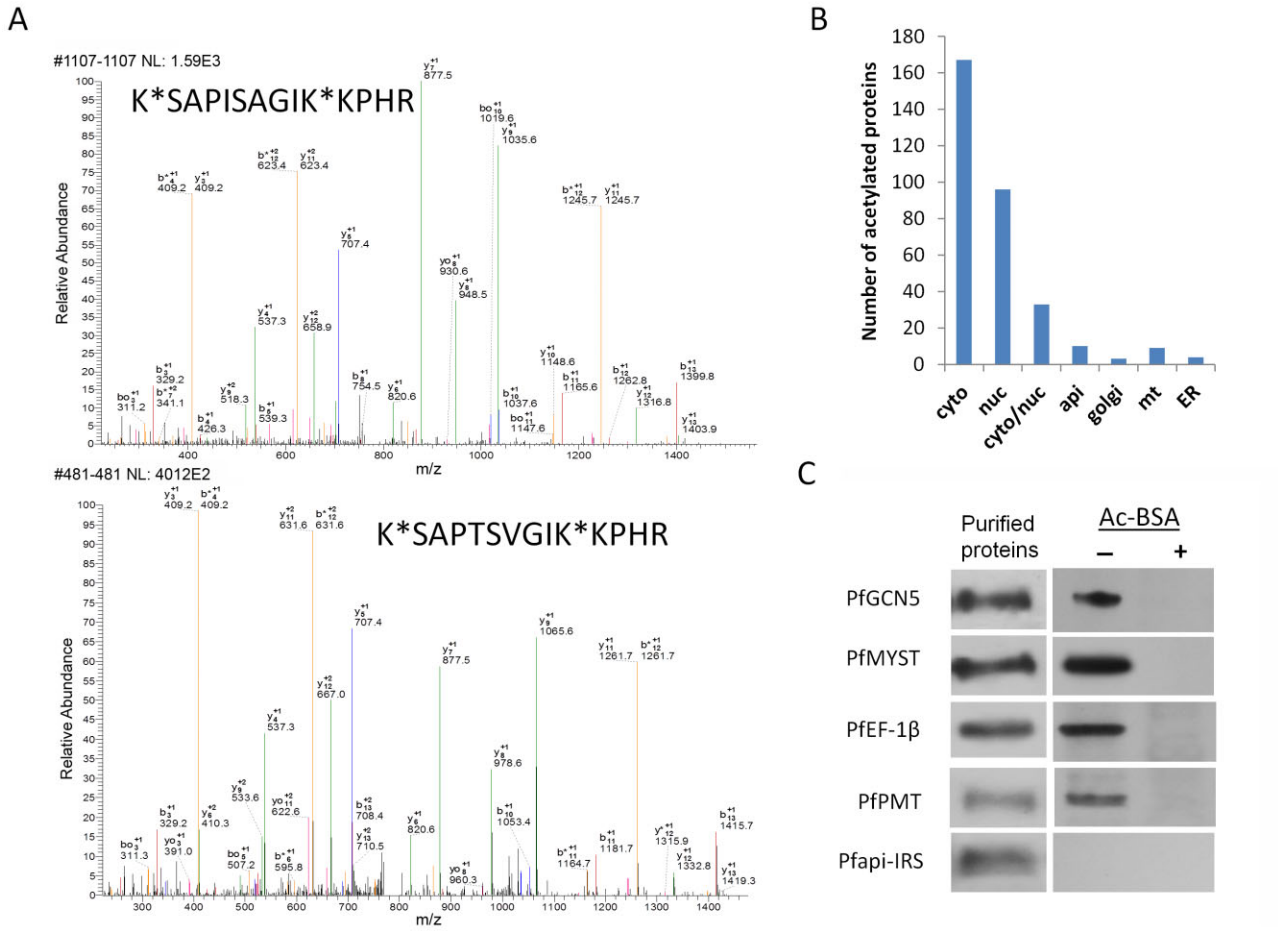

Fig. 2. Identification of protein acetylation in *P. falciparum* trophozoites.
A. Two representative MS results showing the modification of H3 and H3.3 at K28 and K37. Each peptide was fragmented by MS/MS and the fragments observed were consistent with the sequence of the peptide as shown on top of each MS/MS spectrum. Note that b ions are counting from N-terminus and y ions from C-terminus. Overall the MS/MS data unambiguously confirmed that both peptides were diacetylated and they differ by two amino acids.
B. Localization of acetylated proteins in different cellular compartments.
C. Verification of acetylation of five proteins by immunoprecipitation and Western blot with anti-acetyllysine antibodies.

acetylation has been observed in other eukaryotes (Kim *et al.*, 2006; Choudhary *et al.*, 2009; Zhao *et al.*, 2010; Lundby *et al.*, 2012). In the *P. falciparum* acetylome, however, only nine acetylated proteins are predicted to localize in the mitochondrion (Fig. 2B). Another organelle derived from endosymbiosis in the apicomplexan parasites is the apicoplast, a non-photosynthetic plastid (Ralph *et al.*, 2004). Of the ~500 proteins predicted to localize to the apicoplast, 10 acetylated proteins were identified from this study (Fig. 2B). Among them, three belong to translational machinery (two ribosomal proteins and one aminoacyl-tRNA synthetases), one functions in fatty acid synthesis (acyl-CoA-binding protein), and four are conserved *Plasmodium* proteins with unknown functions.

Confirmation of lysine-acetylated proteins

We selected several newly identified lysine-acetylated proteins including two KATs (PfGCN5 and PfMYST) (Miao *et al.*, 2010), phosphatidylethanolamine *N*-methyltransferase (PfPMT) (Pessi *et al.*, 2004), and elongation factor 1 β (PfEF1β) (Mamoun and Goldberg, 2001) for verification of acetylation. These proteins were chosen because they are among the most heavily acetylated proteins and there are specific antibodies or tagged parasite lines for these proteins. We also included apicoplast isoleucine tRNA ligase (Pfapi-IRS) (Istvan *et al.*, 2011) as a negative control since no acetyllysine was detected in this protein. These proteins were subjected to IP and Western analysis (Fig. 2C). The purified proteins were first con-

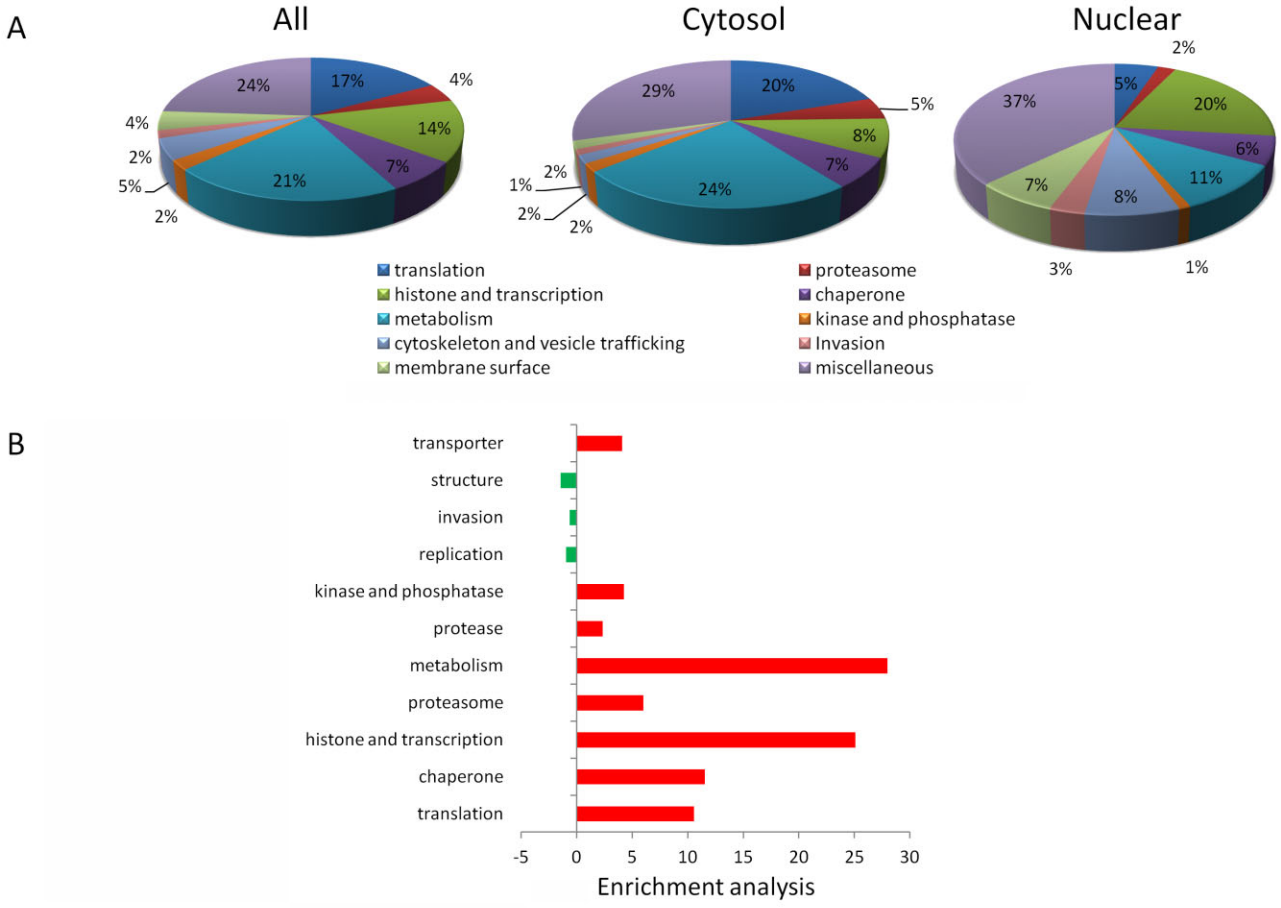

Fig. 3. Acetylation occurs in proteins involving diverse functions in *P. falciparum*.
A. Functional classification of lysine-acetylated proteins from all or cytosolic and nuclear fractions.
B. Enrichment analysis. The red bars indicate the proteins at those functional groups are significantly overrepresented, whereas the green bars indicate under-represented functional groups. *P*-values were calculated using simulations and were then transformed using the negative natural log for visualization.

firmed by their specific antibodies. Anti-acetyllysine antibodies detected the respective proteins, further confirming that they contain acetylated lysine residues, whereas these bands were not detected when acetyl-BSA was included as a competitor (Fig. 2C). In comparison, the negative control protein Pfapi-IRS was detected by specific antibodies, but not the anti-acetyllysine antibodies (Fig. 2C).

Lysine acetylation in proteins with diverse functions

This study identified lysine acetylation in proteins with diverse cellular functions, including histones, regulators of transcription and chromatin structure, splicing, translation, chaperones, cytoskeleton, signalling, metabolic enzymes and proteasome, suggesting that lysine acetylation regulates diverse cellular processes as reported in the acetylome surveys in other organisms (Kim *et al*., 2006; Yu *et al*., 2008; Choudhary *et al*., 2009; Zhang *et al*., 2009; Wang *et al*., 2010; Zhao *et al*., 2010; Finkemeier *et al*., 2011; Weinert *et al*., 2011; Wu *et al*., 2011; Henriksen *et al*., 2012; Jeffers and Sullivan, 2012; Lundby *et al*., 2012). The largest category of lysine-acetylated proteins in both nuclear and cytosol fractions is 'hypothetical proteins' that are conserved in all *Plasmodium* species, which accounts for 24% of the entire identified acetylome. Consistent with the functional distinction between the nucleus and cytoplasm, the second most abundant lysine-acetylated proteins in the nucleus and cytosol belong to the functional categories 'histone and transcription' and 'metabolism' respectively (Fig. 3A). Despite evolutionary conservation of pathways targeted for protein lysine acetylation, detailed analysis revealed substantial divergence of the *Plasmodium* acetylome in both proteins and their acetylation sites. One interesting finding is that the structural protein tubulin is found in other species including *Toxoplasma*, but was absent in the *P. falciparum* trophozoite acetylome. Further distinction of the *Plasmo-*

dium acetylome is indicated in *Plasmodium*-specific proteins such as those involved in haemoglobin digestion, transporters and membrane or surface proteins (Fig. 3A, Tables S1–S3). To determine which functional categories are specially targeted for lysine acetylation, enrichment analysis was performed. Comparison with the genomic representations of these functional categories showed that proteins involved in translation, transcription, metabolism and chaperones were significantly enriched in the *Plasmodium* acetylome, whereas proteins involved in replication, invasion and structure were relatively underrepresented (Fig. 3B). Detailed accounts of the acetylated proteins are provided in the supplemental materials.

Conserved acetylation identified in various metabolic pathways

Lysine-acetylated enzymes were identified in various metabolic pathways including central metabolism, haemoglobin digestion, purine and pyrimidine, phospholipid, polyamine and vitamin B6 and B9 metabolism (Table S1).

Recent studies highlighted a conserved role of lysine acetylation in the regulation of central carbon metabolism in both prokaryotes and eukaryotes (Kim *et al*., 2006; Zhang *et al*., 2009; Wang *et al*., 2010; Zhao *et al*., 2010). Metabolic changes dynamically regulate lysine acetylation and activities of key enzymes in the TCA cycle, the urea cycle and the fatty acid oxidation. Central metabolism in the malaria parasite is different from its host. Living in a glucose-rich environment, the blood-stage *Plasmodium* relies primarily upon glucose fermentation for energy needs. All enzymes of the complete Embden–Meyerhof–Parnas pathway of glycolysis are encoded in the parasite genome and highly expressed during the IDC (Olszewski and Llinas, 2011). Similar to bacteria (Yu *et al*., 2008; Zhang *et al*., 2009; Wang *et al*., 2010), yeast (Henriksen *et al*., 2012), *Toxoplasma* (Jeffers and Sullivan, 2012), *D. melanogaster* (Weinert *et al*., 2011), rat (Lundby *et al*., 2012) and human cells (Kim *et al*., 2006; Choudhary *et al*., 2009; Zhao *et al*., 2010), 10 of the 11 enzymes in this pathway are acetylated with 26 acetylated lysines (Fig. S1, Table S1). Sequence alignment of these enzymes with their orthologues in nine organisms with reported acetylomes showed that 14 of 26 acetyllysines in *P. falciparum* are conserved in at least one of their respective orthologues (Fig. S2). In addition, although the rest of acetyllysine sites are not conserved, they are located in domains that are rich in acetyllysines in the flanking regions of their orthologues, suggesting that acetyllysines might be important for the functions of these enzymes (Fig. S2). Within the TCA cycle, only isocitrate dehydrogenase was found acetylated in this study.

Plasmodium purine and pyrimidine metabolic pathways are distinct from those of the human host and they are extensively pursued for novel drug development (Cassera *et al*., 2011). *P. falciparum* is a purine auxotroph, salvaging host cell purines for synthesis of cofactors and nucleic acids. Unlike purines, pyrimidines exist at only low concentrations in human erythrocytes and *P. falciparum* primarily synthesizes pyrimidines *de novo* (Cassera *et al*., 2011). Eventually, ribonucleotide diphosphate reductase converts purine and pyrimidine nucleotides to deoxyribonucleotides (Wilson *et al*., 1952; Bracchi-Ricard *et al*., 2005). In the purine salvage and pyrimidine synthesis pathways (Cassera *et al*., 2011), six enzymes were lysine-acetylated (Fig. 4). In addition, two enzymes involved in the biosynthesis of polyamines were acetylated.

RNA splicing and translation factors

This study identified 37 lysine-acetylated proteins in this functional group, accounting for 17% of total lysine-acetylated proteins (Fig. 2B). These proteins include ribosome proteins, splicing factors, translation initiation factors, elongation factors and aminoacyl-tRNA synthetases, suggesting that acetylation might play a profound role in regulating RNA splicing and translation in *Plasmodium*. Among them, elongation factor 1-α was heavily acetylated at 8 lysine residues, similar to the observations made in its orthologues in other eukaryotes (human, rat, *Drosophila*, plant and *Toxoplasma*) (Choudhary *et al*., 2009; Finkemeier *et al*., 2011; Weinert *et al*., 2011; Wu *et al*., 2011; Jeffers and Sullivan, 2012; Lundby *et al*., 2012). In addition, K304 is a conserved site among plant (Finkemeier *et al*., 2011; Wu *et al*., 2011), human (Choudhary *et al*., 2009) and *Plasmodium* (Fig. S3). Of the five cytosolic aminoacyl-tRNA synthetases (glutaminyl-tRNA synthetase, valine-tRNA ligase, glutamate-tRNA ligase, seryl-tRNA synthetase and isoleucine-tRNA ligase), isoleucine-tRNA synthetase (Istvan *et al*., 2011) is heavily acetylated at seven lysine residues, which is in stark contrast with the monoacetylation detected in the homologues of human and *Toxoplasma*. This difference may suggest a regulatory mechanism of acetylation for the cytosolic isoleucine-tRNA synthetase in *Plasmodium*. Aminoacyl-tRNA synthetases are potential targets for intervention, and inhibitors of isoleucine-tRNA synthetase and seryl-tRNA synthetase are found to inhibit *P. falciparum* growth (Istvan *et al*., 2011; Hoepfner *et al*., 2012).

Histones, chromatin-associated proteins and transcriptional factors

Among the covalent modifications, histone lysine acetylation is highly abundant and acetylation sites are evolutionarily conserved. This study confirmed 19 of the 29 known acetylation sites, including almost all known acetylation sites in H4, H2A.Z and H2B.X (Miao *et al*., 2006;

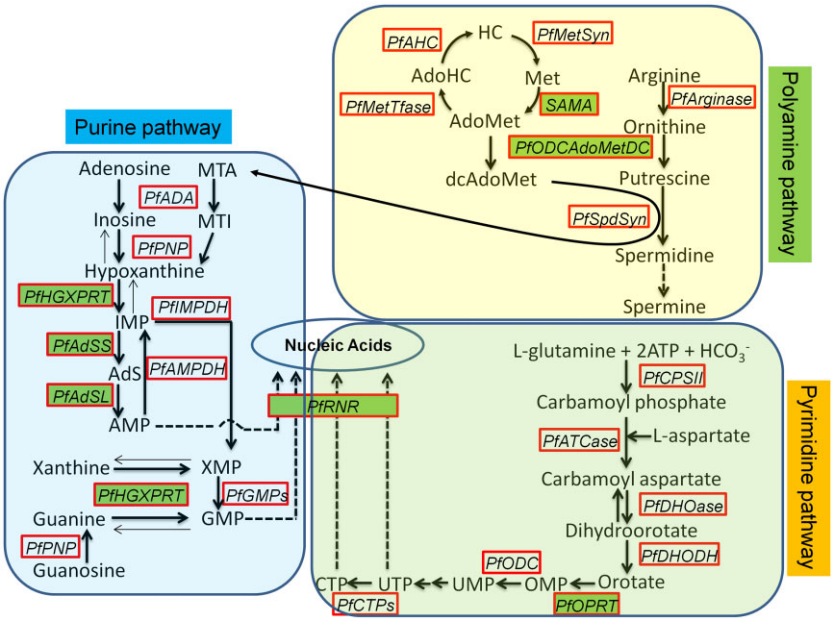

Fig. 4. Acetylation of enzymes in the purine, pyrimidine and polyamine metabolic pathways in *P. falciparum*. Purine pathway: AMP, adenosine 5′-monophosphate; IMP, inosine 5′-monophosphate; XMP, xanthosine 5′-monophosphate; GMP, guanosine 5′-monophosphate; MTA, methylthioadenosine; MTI, methylthioinosine; AdS, adenoylsuccinate; *PfADA, P. falciparum* adenosine deaminase; *PfPNP, P. falciparum purine nucleoside phosphorylase; PfHGXPRT, P. falciparum hypoxanthine-guanine-xanthine phosphoribosyl transferase; PfAMPDA, P. falciparum adenosine 5′-monophosphate deaminase; PfIMPDH, P. falciparum inosine 5′-monophosphate dehydrogenase; PfGMPs, P. falciparum guanosine* 5′-monophosphate synthase; *PfAdSS, adenylosuccinate synthetase; PfAdSL*, adenylosuccinate lyase. Pyrimidine pathway: OMP, orotidine 5′-monophosphate; UMP, uridine 5′-monophosphate; UTP, uridine 5′-triphosphate; CTP, cytidine 5′-triphosphate; *PfCPSII, P. falciparum carbamoyl phosphate synthetase II; PfATCase, P. falciparum* aspartate carbamoyltransferase; *PfDHOase, P. falciparum dihydroorotase; PfDHODH, P. falciparum dihydroorotate dehydrogenase; PfOPRT, P. falciparum orotate phosphoribosyltransferase*; *PfODC, P. falciparum orotidine 5′-monophosphate decarboxylase; PfCTPs, P. falciparum cytidine 5′-triphosphate synthase.* Polyamine pathway: AdoMet, *S*-adenosylmethionine; AdoHC, *S*-adenosylhomocysteine; HC, homocysteine; Met, methionine; dcAdoMet, decarboxylated *S*-adenosylmethionine; *PfSpdSyn, P. falciparum spermidine synthase*; *PfODCAdoMetDC, P. falciparum ornithine decarboxylase/S-adenosylmethionine decarboxylase*; *PfMetTfase, P. falciparum methyltransferase(s); PfAHC, P. falciparum S-adenosyl homocysteinase*; *PfMetSyn, P. falciparum methionine synthase*; *SAMS, P. falciparum S-adenosylmethionine synthase*. The arrows indicate the direction of net flux. The metabolically unfavoured direction is depicted with light arrows on reversible steps. Multiple arrows indicate pathways not shown entirely. The enzymes are boxed and acetylated enzymes are shown in green blocks.

Salcedo-Amaya *et al.*, 2009). However, this study failed to detect known acetylation sites in H2A and K18, K23, K56 in H3, and K9, K14, K18 and K23 in H3.3, which could be due to intrinsic low abundance of acetylation at these sites in trophozoites or low affinity of antibodies to these sites. In addition, five new acetylation sites in histones were detected: H3K36, H2A.ZK36, H3.3 K36, and two sites (K23, K26) in CenH3. Unlike acetylation in core histones, the functional significance of lysine acetylation in minor or variant histones is less understood.

Besides histones, quite a number of proteins associated with chromatin biology were found to be lysine-acetylated. The two KATs PfGCN5 and PfMYST contain 7 and 11 acetyllysines respectively (Fig. 5, Table S1). In addition, the Elp3-like KAT and another putative histone acetyltransferase were also acetylated. In PfMYST, acetylated lysine 428 and its corresponding lysine residues in yEsa1 K262, SAS2 K168, hMOF K274 and TgMYST-A K288 were also found acetylated, and this lysine is located in the active domain of these enzymes and its acetylation is essential for activity (Choudhary *et al.*, 2009; Jeffers and Sullivan, 2012; Yuan *et al.*, 2012). Similar to the observation in metazoan acetylomes, acetylation in *Plasmodium* also potentially targets protein complexes. A member of the PfGCN5 KAT complex, PfADA2, contains nine acetyllysines (Fan *et al.*, 2004a). Two PHD domain proteins potentially associated with the PfGCN5 complex (PF10_0079 and PF14_0315) were also abundantly acetylated with 18 and 14 acetyllysines respectively (Fig. 5). Interestingly, two PHD domain containing proteins (TGME49_024260 and TGME49_034700) were also found acetylated at six and four lysines in *T. gondii* respectively (Jeffers and Sullivan, 2012). In addition to these KATs and related proteins, other chromatin-associated proteins were found acetylated including two high mobility group proteins (PfHMGB1 and PfHMGB2), PCNA, a SWI/SNF factor, two bromodomain-containing proteins and a putative histone lysine methyltransferase (Table S1).

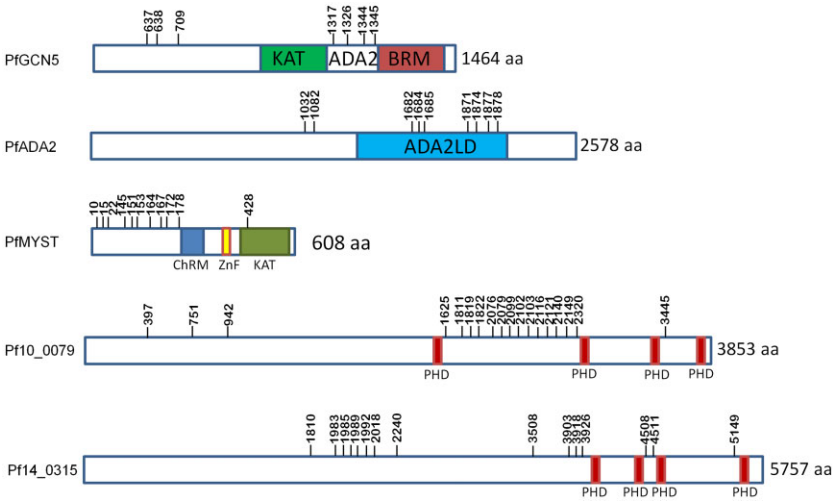

Fig. 5. Acetylation sites in *P. falciparum* KATs and their associated proteins. The structures of two key KATs, PfGCN5 and PfMYST, and their associated proteins, PfADA2, Pf10_0079, Pf14_0315 are depicted. The acetylated sites are shown with short bars with numbers indicating the positions of lysine residues. KAT, lysine acetyltransferase activity domain; ADA2, ADA2-binding domain; BRM, bromodomain; ADA2LD, ADA2 like-domain; ChRM, chromodomain; Znf, zinc finger; PHD, PHD domain.

Other protein groups

Chaperones are a group of proteins that are enriched in lysine acetylation with 16 lysine-acetylated proteins identified, which are similar to the profiles of acetylated chaperones documented in human, rat and *Drosophila* (Kim *et al*., 2006; Choudhary *et al*., 2009; Zhao *et al*., 2010; Weinert *et al*., 2011; Lundby *et al*., 2012), suggesting a high level of conservation in the acetylation network of chaperones. It is worth to mention that PfHsp90 contains five acetyllysine sites and two proteins (Hsp101 and PTEX150) of the recently identified novel protein export complex PTEX (*Plasmodium* Translocon of EXported proteins) (de Koning-Ward *et al*., 2009) were also acetylated.

This study revealed 12 lysine-acetylated proteins in protein ubiquitylation and sumoylation pathways, including six subunits of the 26S proteasome complex, a deubiquinating/deneddylating enzyme (Artavanis-Tsakonas *et al*., 2006), a ubiquitin-ribosomal fusion protein, SUMO, a bacteria-like proteasomal predecessor PfhslV (Ramasamy *et al*., 2007), a putative ubiquitin ligase, and a Dsk2-like proteasomal regulator. It contrast, none of the proteins in the human proteasome complex were found acetylated (Choudhary *et al*., 2009), highlighting a major difference in the ubiquitylation system in *Plasmodium* compared with its host.

As seen in the *Toxoplasma* tachyzoite acetylome, hypothetical proteins comprise the largest category of lysine-acetylated proteins in *Plasmodium* (Fig. 3A, Table S1). Furthermore, more than 20 acetylated proteins lack homologues in metazoan species. Among them are membrane surface proteins important for cytoadherence and invasion.

Acetylation of KATs and their activities

In order to understand the effect of lysine acetylation on the enzymatic activities of the two KATs PfGCN5 and PfMYST, GFP-tagged PfGCN5 and PfMYST were purified from trophozoite stage parasites of transgenic parasite lines treated with or without HDAC inhibitor (TSA and nicotinomide) treatment. Anti-GFP antibodies showed that similar amounts of purified PfGCN5 or PfMYST were used for *in vitro* HAT assays (Fig. 6). Anti-acetyllysine antibodies revealed that HDAC inhibitor treatment resulted in higher levels of acetylation in PfGCN5 and PfMYST (Fig. 6). When core and nucleosomal histones were used as the substrates, increased acetylation of PfGCN5 did not significantly change its HAT activities. However, higher acetylation of PfMYST was associated with a significant decrease of its HAT activity on H4 in both core and nucleosomal histones (Fig. 6). It is noteworthy that autoa-

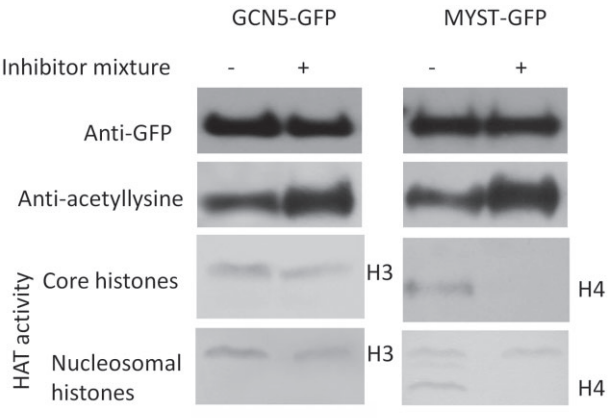

Fig. 6. Effect of lysine acetylation on HAT activities of PfGCN5 and PfMYST. GFP-tagged PfGCN5 and PfMYST were immunoprecipitated from *P. falciparum* trophozoites cultured without or with the inhibitor mixture (TSA and nicotinamide). Blots with anti-GFP antibodies indicate approximately equal amounts of purified PfGCN5 or PfMYST used in the HAT assays. The overall acetylation levels of the purified proteins were estimated by Western blots with anti-acetyllysine antibodies. HAT activities of purified PfGCN5 and PfMYST were determined using core histones and nucleosomal histones.

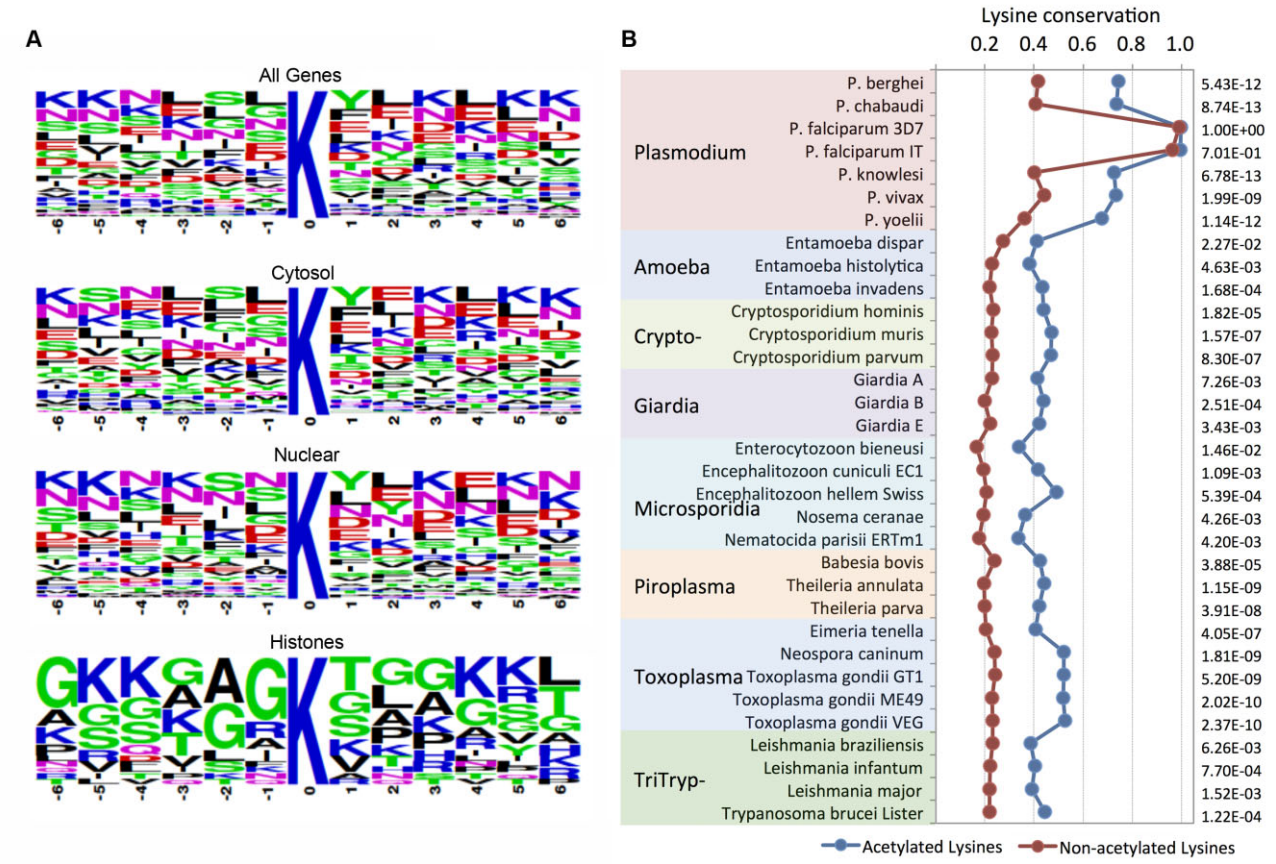

Fig. 7. Acetylation motifs and conservation of acetylation sites.
A. Relative abundance of each amino acid residues surrounding sites of acetylated lysine in all acetylated proteins, cytosolic proteins, nuclear proteins and histones.
B. Lysine conservation of *P. falciparum* acetylated lysines in other pathogen species. Acetylated lysines are significantly more conserved than non-acetylated lysines.

cetylation of PfGCN5 and PfMYST in the *in vitro* reactions was not obvious, although autoacetylation of recombinant PfGCN5 HAT domain was previously detected (Fan *et al*., 2004b).

Acetylation motifs and preference

To elucidate the acetylation motifs in *Plasmodium*, we analysed the composition of amino acids surrounding the acetylated lysines (Fig. 7A). Similar to other studies, we could not obtain definitive motifs but only identified a set of patterns. In metazoans, acetylated lysines of nuclear and cytosolic proteins tend to be flanked by F at the –2 position, Y at the +1 position and additional stretches of Ks on both sides (Choudhary *et al*., 2009; Weinert *et al*., 2011). However, the sequence motifs differ by subcellular compartments. In the rat acetylomes, a strong preference for glycine in position –1 and proline in position +1 is observed on nuclear proteins, whereas cytoplasmic proteins are enriched with glutamate in the vicinity of the acetylation site (Lundby *et al*., 2012). In *P. falciparum*, the acetylation motifs in histones are better defined and a GK motif is highly conserved (Fig. 7A). However, at +1 position T is the most commonly found, compared with the most common residues of A and S in human (Choudhary *et al*., 2009) and *Toxoplasma* (Jeffers and Sullivan, 2012) histones respectively. Consistently in *Plasmodium* there is a relative enrichment of lysines in the flanking sequences, but other motifs are less clearly defined. Whereas Y at +1 position is conserved in cytosolic and nuclear proteins, S instead of F is the most common at –2 position.

Evolutionarily conserved acetylation sites may indicate functional significance of acetylation on protein functions. To determine whether acetylation sites are evolutionarily conserved, we compared the degree of conservation in acetylated versus non-acetylated lysine residues across 32 protozoan species and microsporidia. Orthologues of acetylated *Plasmodium* proteins were retrieved from genome sequences of EupathDB using BLASTP, and the conservation of acetylated lysines was determined by

sequence alignment. The results showed that acetylated lysines in *Plasmodium* are significantly more conserved comparing to non-acetylated lysines across a wide range of protozoan parasites and microsporidia (Fig. 7B).

Conservation of acetylation in the Apicomplexa

Due to the close phylogenetic relationship between *Plasmodium* and *Toxoplasma*, we performed a qualitative comparison of the acetylome data obtained to date for each parasite. In each parasite, the functional breakdown of acetylated proteins was similar, with 71 proteins detected as acetylated in both species (Table 1). Among some of the commonalities, histone acetylation, along with the acetylation of KATs and components of KAT complexes, are shared features between the parasite acetylomes. Moreover, the acetylation marks largely appear to cluster within the same regions of both *P. falciparum* and *T. gondii* KAT homologues (Fig. S4), suggesting that lysine acetylation may regulate the formation and function of these acetylation complexes. Consistent with this idea, acetylation marks are enriched in the ADA2-interaction domain of both species' GCN5 KATs. PfMYST and the two MYSTs in *T. gondii* are heavily acetylated within the N-terminal extension upstream from the KAT domain, a region in which the function has yet to be defined. Acetylation of K428 within the KAT domain of PfMYST is an example of an acetyl mark that appears conserved across eukaryotes. The equivalent lysine is acetylated in TgMYST-A as well as in yeast and human MYST proteins; this particular PTM has been demonstrated to regulate nucleosome interaction and acetylation (Sun *et al*., 2011; Yuan *et al*., 2012). Also noteworthy is that acetylated proteins exist within all cellular compartments beyond the nucleus, including the mitochondrion and apicoplast, although the types of proteins detected as acetylated within these compartments were not absolutely conserved. Phosphoglycerate kinase was found to be acetylated in each parasite's apicoplast, and four mitochondrial proteins were acetylated in both species (fumarase, isocitrate dehydrogenase, Hsp60 and Hsp70). AP2 factors were found to be acetylated in both species, but it is not possible at present to judge if the AP2s in question are orthologous. Other common features can be identified with respect to metabolic proteins, including components of translation. For example, three amino-acyl tRNA transferases are acetylated in both apicomplexan parasites, but the lysines targeted are not the same. Acetylation of ribosomal proteins is common and includes three acetyl-sites on homologous residues. Several orthologous heat shock proteins are acetylated in both parasites, with two of these acetyl marks being absolutely conserved. A large number of metabolic enzymes were detected as acetylated in both *Plasmodium* and *Toxoplasma*, with 13 proteins common to both acetylomes and six of the acetylated lysines being homologous. Finally, there are a number of hypothetical proteins with no known function conserved in both parasites that are commonly subject to acetylation (Table 1).

Discussion

We have screened acetylated proteins during *Plasmodium* IDC and identified 230 lysine-acetylated proteins with 421 acetylation sites. These acetylated proteins belong to diverse functional groups, including those that perform evolutionarily conserved functions as well as *Plasmodium* specific. Compared with acetylome from bacteria, yeast, *Drosophila*, rat and human cells, the number of protein lysine acetylation detected in *Plasmodium*, as well as in another apicomplexan parasite, *T. gondii* (with 753 lysine acetylation sites in 486 proteins in tachyzoites), is more similar to those in prokaryotes than in metazoans (Jeffers and Sullivan, 2012). A more detailed comparison revealed some interesting parallels as well as dramatic differences between the *Plasmodium* and *Toxoplasma* acetylomes. The unconserved features of each parasite acetylome may be due to differences in technique or current coverage, but may also reflect adaptations of the parasites to selective pressures from different living environments, suggesting adaptive regulation of parasite-specific pathways by acetylation. The similarity in the types of proteins that are acetylated, or in some cases even the lysine residue targeted for acetylation, likely underscores a vital biological role for this modification across Apicomplexa.

Overall, the acetylation motifs in most organisms studied so far are not well defined. This, in turn, limits the power of algorithms for proteome-wide prediction of acetylation substrates (Basu *et al*., 2009). Furthermore, the acetylation motifs appear to differ between proteins of different cellular compartments (Lundby *et al*., 2012). This is likely due to differential distributions of various KATs, which may have different sequence preferences. Indeed, prediction algorithms based on the preferred sequence features of different KAT families improve the power of prediction (Li *et al*., 2012). In *Plasmodium*, the cytosolic and nuclear acetylomes also show tremendous differences in the acetylation motifs, and this distinction is likely attributed to the different subcellular localizations of the KATs. In particular, PfMYST is abundantly present in both cytosol and nucleus, whereas PfGCN5 is restricted to the nucleus (Miao *et al*., 2010). Similar localization patterns have been observed for these KATs in *T. gondii*. Except histones, the substrate targets and sequence preferences of these KATs are not known. Therefore, future studies aimed at identifying the substrates of individual KATs are needed to elucidate the functional division of KATs.

Table 1. Acetylated proteins shared between *Plasmodium falciparum* (Pf3D7) and *Toxoplasma gondii*.

*Pf*3D7 ID	Protein name	*T. gondii* ID	Protein name
MAL8P1.69	14-3-3 protein, putative	TGME49_063090	14-3-3 protein, putative
MAL8P1.142	20S proteasome beta subunit	TGME49_080710	Proteasome A-type and B-type domain-containing protein
MAL7P1.300	40S ribosomal protein S29, putative	TGME49_042340	40S ribosomal protein S29, putative
PFC1020c	40S ribosomal protein S3A, putative	TGME49_032710	40S ribosomal protein S3a, putative
PF14_0141	60S ribosomal protein L10, putative	TGME49_088720	60S ribosomal protein L10, putative
PFF0885w	60S ribosomal protein L27a, putative	TGME49_110490	Ribosomal protein L22, putative
PF10_0272	60S ribosomal protein L3, putative	TGME49_027360	60S ribosomal protein L3, putative
PF13_0346	60S ribosomal protein L40/UBI, putative	TGME49_089750	Ubiquitin/ribosomal protein CEP52 fusion protein, putative
PFI0755c	6-Phosphofructokinase (PFK9)	TGME49_026960	Phosphofructokinase, putative
PFF1350c	Acetyl-CoA synthetase, putative	TGME49_066640	Acetyl-coenzyme A synthetase, putative
PF13_0131	Acetyltransferase, GNAT family, putative	TGME49_030060	Acetyltransferase domain-containing protein
PFL2215w	Actin I (ACT1)	TGME49_009030	Actin
PF11_0197	Acyl-CoA-binding protein, putative	TGME49_034510	Acyl-CoA-binding protein, putative
PF14_0241	Basic transcription factor 3b, putative	TGME49_057090	NAC domain containing protein
PFL0635c	Bromodomain protein, putative	TGME49_064640	Bromodomain domain-containing protein
PF14_0510	Co-chaperone p23 (P23)	TGME49_121520	P23 co-chaperone, putative
PFF0835w	Conserved *Plasmodium* protein	TGME49_032440	Hypothetical protein
MAL8P1.95	Conserved *Plasmodium* protein	TGME49_060440	46 kDa FK506-binding nuclear protein, putative
PFL0640w	Conserved *Plasmodium* protein	TGME49_008800	Hypothetical protein, conserved
PFF1295w	Conserved protein, unknown function	TGME49_115250	Melanocyte proliferating gene 1, putative
PFC0170c	Dihydrolipoamide acyltransferase, putative	TGME49_119920	Dihydrolipoamide branched chain transacylase, E2 subunit, putative
PF13_0305	Elongation factor 1-alpha	TGME49_086420; TGME49_094800	Elongation factor 1-alpha, putative; Elongation factor 1-alpha, putative
PFI0645w	Elongation factor 1-beta (EF-1beta)	TGME49_026410	Elongation factor 1-beta, putative
PF14_0486	Elongation factor 2	TGME49_005470	Elongation factor 2, putative
PF10_0155	Enolase (ENO)	TGME49_068850	Enolase 2
PF14_0655	Eukaryotic initiation factor 4A (eIF4A)	TGME49_050770	Eukaryotic translation initiation factor 4A
PF08_0071	Fe-superoxide dismutase (FeSOD)	TGME49_116310	Superoxide dismutase
PF14_0425	Fructose-bisphosphate aldolase	TGME49_036040	Fructose-1,6-bisphosphate aldolase
PFI1340w	Fumarate hydratase, putative	TGME49_067330	Fumarase, putative (mitochondrial localization)
PF14_0511	Glucose-6-phosphate dehydrogenase-6-Phosphogluconolactonase (G6PDH)	TGME49_078830	Glucose-6-phosphate dehydrogenase, putative
PF08_0132	Glutamate dehydrogenase, putative (GDHc)	TGME49_049390	NAD-specific glutamate dehydrogenase, putative
PF13_0257	Glutamate–tRNA ligase, putative	TGME49_063870	Glutamyl-tRNA synthetase, putative
PF14_0598	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	TGME49_089690; TGME49_069190	Glyceraldehyde-3-phosphate dehydrogenase
PF11_0183	GTP-binding nuclear protein ran/tc4 (RAN)	TGME49_048340	GTP-binding nuclear protein RAN/TC4
PF10_0153	Heat shock protein 60 (HSP60)	TGME49_047550	Heat shock protein 60 (mitochondrial localization)
PF08_0054	Heat shock protein 70 (Hsp70)	TGME49_073760	Heat shock protein 70, putative (mitochondrial localization)
PFI0875w	Heat shock protein 70 (Hsp70-2)	TGME49_111720	Heat shock protein 70, putative
PF07_0029	Heat shock protein 90 (HSP90)	TGME49_088380	Heat shock protein 90
PFF1155w	Hexokinase (HK)	TGME49_065450	Hexokinase
PFL0145c	High mobility group protein (HMGB1)	TGME49_010410	High mobility group protein
MAL8P1.72	High mobility group protein (HMGB2)	TGME49_010410	High mobility group protein
PF08_0034	Histone acetyltransferase GCN5 (GCN5)	TGME49_043440	Histone acetyltransferase GCN5, putative
PF11_0192	Histone acetyltransferase, putative (MYST)	TGME49_118330; TGME49_007080	MYST-family histone acetyltransferase-A; histone acetyltransferase
PFC0920w	Histone H2A variant, putative (H2A.Z)	TGME49_100200	Histone H2A
PF07_0054	Histone H2B variant, putative (H2Bv)	TGME49_009910; TGME49_105160; TGME49_051870	Histone H2B variant 1; histone H2B, putative; histone H2B, putative
PFF0510w	Histone H3 (H3)	TGME49_061240	Histone H3
PFF0865w	Histone H3 variant, putative (H3.3)	TGME49_018260	Histone H3.3 variant
PF11_0061	Histone H4 (H4)	TGME49_039260	Histone H4, putative
PFE1370w	hsp70 interacting protein, putative	TGME49_032660	58 kDa phosphoprotein, putative
PF14_0324	Hsp70/Hsp90 organizing protein (HOP)	TGME49_052220	Hsc70/Hsp90-organizing protein, putative
PF11_0189	Insulinase, putative	TGME49_014490	M16 family peptidase, putative
PF13_0242	Isocitrate dehydrogenase (NADP), mitochondrial precursor (IDH)	TGME49_113140	Isocitrate dehydrogenase, putative (mitochondrial localization)
PF13_0179	Isoleucine-tRNA ligase, putative	TGME49_007640	Isoleucine-tRNA synthetase, putative
PF13_0141	L-lactate dehydrogenase (LDH)	TGME49_032350	Lactate dehydrogenase
PF14_0439	M17 leucyl aminopeptidase (LAP)	TGME49_090670	Cytosol aminopeptidase
PF10_0036	*N*-acetyltransferase, putative	TGME49_019760	N-terminal acetyltransferase complex subunit ARD1, putative
PFE0630c	Orotate phosphoribosyltransferase (OPRT)	TGME49_059660	Orotate phosphoribosyltransferase, putative
PF13_0234	Phosphoenolpyruvate carboxykinase (PEPCK)	TGME49_089650	Phosphoenolpyruvate carboxykinase
PFI1105w	Phosphoglycerate kinase (PGK)	TGME49_022020; TGME49_118230	Phosphoglycerate kinase, putative; phosphoglycerate kinase, putative (apicoplast localization)
PF11_0208	Phosphoglycerate mutase, putative (PGM1)	TGME49_097060	Phosphoglycerate mutase 1, putative
PFL1170w	Polyadenylate-binding protein, putative (PABP)	TGME49_024850	Polyadenylate-binding protein, putative
PFF0320c	Polypyrimidine tract-binding protein, putative	TGME49_090660	Polypyrimidine track-binding protein, putative
PFF1025c	Pyridoxine biosynthesis protein PDX1 (PDX1)	TGME49_037140	Ethylene inducible protein, putative
PFF1300w	Pyruvate kinase (PyrK)	TGME49_056760	Pyruvate kinase, putative
PF08_0019	Receptor for activated c kinase (RACK)	TGME49_016880	Receptor for activated C kinase, RACK protein, putative
PFL1720w	Serine hydroxymethyltransferase (SHMT)	TGME49_034190	Glycine hydroxymethyltransferase, putative
PF07_0073	Seryl-tRNA synthetase, putative	TGME49_051690	seryl-tRNA synthetase, putative
PF10_0266	Small subunit rRNA processing stabilizing factor, putative	TGME49_111410	U3 small nucleolar ribonucleoprotein protein MPP10, putative
PFF1185w	SNF2 helicase, putative (ISWI)	TGME49_036970	SNF2 family N-terminal domain containing protein
PF11_0331	TCP-1/cpn60 chaperonin family, putative	TGME49_029990	TCP-1/cpn60 family chaperonin, putative
PF10_0143	Transcriptional co-activator ADA2 (ADA2)	TGME49_017050	Transcriptional co-activator ADA2-A

Lysine acetylation, which uses acetyl-CoA, is tightly linked to the cellular metabolic status and energy flux. It is thus not surprising that lysine acetylation of key enzymes in the central carbon metabolic pathways is evolutionarily conserved from bacteria to human (Kim *et al*., 2006; Zhang *et al*., 2009; Wang *et al*., 2010; Zhao *et al*., 2010). Accordingly, concentrations of metabolic fuels directly regulate the acetylation status and activities of metabolic enzymes (Wang *et al*., 2010; Zhao *et al*., 2010). Consistent with the essential role of mitochondrion in energy metabolism, a large number of mitochondrial proteins are found acetylated in other species (Kim *et al*., 2006; Choudhary *et al*., 2009; Zhao *et al*., 2010; Lundby *et al*., 2012). Similarly, enzymes of various metabolic pathways make up the most abundant group of acetylated proteins in the *Plasmodium* cytoplasmic acetylome. However, the central carbon metabolism pathways in *Plasmodium* are very divergent from the canonical pathways. The blood-stage malaria parasites, living in a glucose-rich environment, rely primarily on glucose fermentation with the formation of lactate rather than oxidative phosphorylation for its energy need. Likewise, protein lysine acetylation is highly abundant in the glycolysis pathway but not in the TCA cycle, and there is a relative dearth of acetylated proteins in *Plasmodium* mitochondrion. It is also noteworthy that proteins of many pathways unique to the malaria parasite, such as purine salvage and phospholipid biosynthesis, are also abundantly acetylated. Currently, many of these pathways are actively pursued for the development of novel antimalarial drugs.

Histone acetylation is an important epigenetic mark for euchromatin in gene regulation in malaria parasites (Salcedo-Amaya *et al*., 2009). In addition to histone acetylation, this study identified a large number of proteins potentially involved in chromatin biology and transcription. The two KATs PfGCN5 and PfMYST play essential roles in gene expression, cell cycle progression, and DNA repair (Cui *et al*., 2007; Miao *et al*., 2010). These two KATs are among the most heavily acetylated proteins in the nuclear acetylome. In addition, manipulation of the acetylation levels of PfMYST resulted in reduced HAT activity of this enzyme on histone H4. Moreover, other members of the PfGCN5 complex, including PfADA2 and two PHD-domain proteins, are also heavily acetylated. This study identified the highest level of acetylation in a putative KAT complex.

Proteins are subjected to a wide array of post-translational modifications and cross-talk is evident among these modifications (Yang and Seto, 2008; Soufi *et al*., 2012). The same proteins could bear different modifications with potentially different functional consequences. For example, of the 230 proteins identified in our acetylome, 133 (58%) were also detected in a screen for phosphoproteins (Treeck *et al*., 2011). In addition, 17 of the 73 ubiquitylated proteins identified in *P. falciparum* were identified in our acetylome (Ponts *et al*., 2008). Similarly, 29 of the 120 sumoylated proteins were also found to be acetylated in *T. gondii* (Jeffers and Sullivan, 2012). This contrasts with only one protein found to be both acetylated and sumoylated in *P. falciparum*, probably due to the small number of sumoylated proteins (23) identified in this parasite (Lopez-Rubio *et al*., 2007). Since ubiquitylation, sumoylation and acetylation all modify lysine residues, the same lysine may be opted for one of these modifications, which will have drastically different influences on the functions of the proteins (Yang and Seto, 2008; Denuc and Marfany, 2010).

In conclusion, the *Plasmodium* parasites have evolved an extensive protein lysine acetylation network. Yet, the data set reported here and most comparisons are only qualitative rather than quantitative. The biological consequence of protein lysine acetylation in these parasites still awaits future characterization.

Experimental procedures

Parasite culture

Plasmodium falciparum 3D7 was cultured as described previously (Trager and Jensen, 1976). Synchronization of asexual stages was performed by two rounds of sorbitol treatment at the ring stage. For the time-course studies, schizonts were purified by Percoll gradient centrifugation (Miao and Cui, 2011) and mixed with fresh RBCs. Parasites were harvested at 10, 20, 30 and 40 h later to represent ring, early trophozoite, late trophozoite and schizont stages respectively.

Manipulation of HAT and HDAC activities

To determine the effect of manipulation of KAT activities on overall acetylation of parasite proteins, we compared protein acetylation among three parasite lines: one with overexpression of a full-length active PfMYST (F1-GFP), one with overexpression of a truncated inactive version of PfMYST (F1C3-GFP) missing 81 amino acids in the C-terminus, and the GFP-control line (Miao *et al*., 2010). The transgenes were controlled by the *hsp86* promoter and a single copy of the expression cassette was integrated at the *attB* locus of the $3D7^{attB}$ strain (Miao *et al*., 2010). Parasites were cultured and harvested at 30 h post invasion. To determine the effect of HDAC inhibitors on protein lysine acetylation in the parasites, 3D7 parasites at ring stage (15 h post invasion) were treated with TSA at $1 \times$ to $4 \times IC_{50}$ of TSA (16–64 nM) and nicotinamide at 5, 10 and 15 mM, which were determined earlier for *P. falciparum in vitro* culture (Prusty *et al*., 2008; Patel *et al*., 2009). After incubation for 15 h, parasites were harvested and protein lysates were prepared and used for Western analysis. To alter the levels of acetylation in PfMYST and PfGCN5, parasite lines with GFP-tagged endogenous PfMYST and PfGCN5 were cultured in the presence or absence of 16 nM TSA plus 5 mM nicotinamide for 15 h.

Parasites were then harvested and GFP-tagged proteins were purified as described below. The HAT activities were measured by a previously described method using bovine core histones and nucleosomal histones as the substrates (Miao *et al.*, 2010).

Preparation of protein lysates

Parasites at the trophozoite stage were treated with 0.05% saponin to lyse the RBCs. Released parasites were pelleted by centrifugation and washed twice with cold phosphate-buffered saline (PBS). To prepare the cytoplasmic and nuclear fractions, parasite pellet was resuspended in five volumes of buffer A [10 mM HEPES, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM EDTA and protease inhibitor cocktail], incubated on ice for 15 min to allow cells to swell, and ground for 40 times using a Dounce homogenizer with a loose pestle. The homogenate was first centrifuged at 700 *g* for 20 min at 4°C, and the supernatant was centrifuged at 10 000 *g* for 10 min to obtain the cytoplasmic extract. The pellet was resuspended in three volumes of buffer B (20 mM HEPES, pH 7.8, 20% glycerol, 200 mM KCl, 0.5 mM DTT, 0.5 mM EDTA, 0.5% NP40 and protease inhibitor cocktail) and dounced for 40 times with a tight pestle. The homogenate was centrifuged at 10 000 *g* for 10 min to obtain the nuclear extract.

Preparation of acetylated peptides

Proteins in the cytoplasmic and nuclear lysates were precipitated by adding four volumes of ice-cold acetone. Precipitated proteins were re-dissolved in 6 M urea/2 M thiourea/10 mM HEPES, pH 8.0, reduced by 1 mM DTT, alkylated with 5.5 mM idoacetamide, and digested with endoproteinase Lys-C (1:100) first and trypsin (1:100) after fourfold dilution in distilled water. The resulting peptides were purified using Sep-Pak C_{18} peptide purification cartridges (Waters Corporation, Milford, MA) and eluted in 50% acetonitrile. The eluates were dried and re-dissolved in an IP buffer (50 mM MOPS, pH 7.2, 10 mM sodium phosphate, 50 mM NaCl and 0.5% NP40), and incubated with agarose-conjugated anti-acetyllysine antibodies (ImmuneChem, Burnaby, British Columbia, Canada) overnight at 4°C. The peptide-bound agarose resin was washed four times with the IP buffer and twice with distilled water. The acetylated peptides bound to the agarose resin were eluted by 0.1% TFA and the final eluates were dried to 5–10 μl using a speedvac.

MS analysis and data processing

The purified acetylated peptide mixture was separated using a two-dimensional (2D) nano liquid chromatography (LC) system (Eksigent Technologies, Dublin, CA) with an Agilent Zorbax SB300-C8 trap and eluted by a reverse phase gradient onto a 0.075 mm × 120 mm emitter packed in-house with the Magic C_{18} material (5 μm, 300 A pore) (Michrom Bioresources, Auburn, CA). Mobile phase solution consisted of a water and 0.1% formic acid aqueous phase and a 0.1% formic acid in 50% acetonitrile:ethanol organic phase. The gradient ran from 1 to 60 min and from 10% to 35% organic phase with a 95% wash and eluted into a Thermo Finnigan LTQ mass spectrometer. In these LC/MS experiments, a full MS scan was acquired in parallel with data-dependent MS/MS scans of the top five most abundant m/z peaks. MS/MS was performed with wideband activation, dynamic exclusion of 1 for 60 s with a list of 300 m/z and a width of ± 1.5/0.5 m/z, collision energy of 35% and noise level of 3000 NL. The peak list was generated using Xcalibur (2.0.5) (Thermo Scientific). The LC/MS/MS data were searched with Bioworks 3.0 (Thermo Scientific) against a *P. falciparum* 3D7 database (3D7.version.2.0, *P. falciparum* Genome Sequencing Consortium at http://www.sanger.ac.uk/resources/downloads/protozoa/plasmodium-falciparum.html) with reversed protein decoys. Search parameters included trypsin digestion (C-terminal K and R cleavage) full cleavage with four missed sites (non-specific cleavages not permitted), amino acid length of 6–100 with tolerance of 1.4 Da, dynamic modifications of methionine methylation (+14 Da), cysteine carboxyamidomethylation (+57 Da) and lysine or arginine acetylation (+42 Da). Mass tolerance for fragment ions is ± 0.5 m/z amu (atomic mass unit). Peptides were filtered by a Ranked Preliminary Score of 1, probability of 1e-3, and minimum 20% theoretical ions observed. The resulting data set was searched against the human protein database to filter out potentially contaminating human proteins. In addition, since acetylation hinders trypsin digestion after the acetylated lysine residue, the data set was further filtered to include peptides with only internally acetylated lysines. Finally, peptides of interest were independently identified manually. The raw spectrum of each acetylated peptide is available at http://ento.psu.edu/research/labs/liwang-cui.

IP and Western blots

Immunoprecipitations were performed to verify the acetylation of individual proteins. GFP monoclonal antibodies (Roche Diagnostics, Indianapolis, IN, USA) were used to pull down respective proteins from transgenic parasite lines carrying GFP-tagged PfGCN5 (Miao and Cui, 2011), PfMYST (Miao *et al.*, 2010) and PFL1210w (Pfapi-IRS) (Istvan *et al.*, 2011). Polyclonal antibodies against PfEF-1β (Mamoun and Goldberg, 2001) and PfPMT (Pessi *et al.*, 2004) were used to pull down respective proteins in 3D7 parasites. For IP, ~ 5×10^9 trophozoites were harvested and lysed in five volumes of PA150 buffer (150 mM KCl, 20 mM Tris-HCl, pH 7.7, 3 mM $MgCl_2$, 0.5 mM DTT, 0.1% Tween 20) containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The lysate was centrifuged for 10 min at 20 000 *g* and the supernatant was incubated with 5 μg of monoclonal anti-GFP antibody (Roche) or 30 μl of rabbit anti-EF-1β and PfPMT antisera on ice for 1 h with occasional mixing. Protein A agarose (30 μl packed bead volume) was added to the lysate and incubated for 1 h. The agarose beads were washed four times with PA150, and bound proteins were either eluted with elution buffer for analysis of activities or resuspended in SDS-PAGE loading buffer for immunoblotting. Eluted proteins in the loading buffer and parasites samples collected from the four time points during IDC were separated in a 12% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with anti-acetyllysine antibodies (ImmuneChem) as primary antibodies

in the presence or absence of acetylated BSA (Kim *et al*., 2006). Secondary horseradish peroxidase-conjugated antibodies were used at 1:3000. Proteins were visualized using an ECL Kit (Life Technologies, Grand Island, NY, USA).

Bioinformatic analysis

Analysis of the amino acid sequences surrounding the acetylation sites was performed using WebLogo (Crooks *et al*., 2004). Acetylated proteins were classified according to the gene ontology (GO) annotation in PlasmoDB 9.0 (http://www.plasmodb.org) (Bahl *et al*., 2003) to include molecular function and cellular localization data. Domain structures of certain proteins were extracted from PlasmoDB 9.0. To determine the degree of evolutionary conservation of acetylation, we first used BLASTP to compare acetylated protein sequences of *P. falciparum* 3D7 against all protein sequences in EupathDB (http://www.eupathdb.org), which includes six *Plasmodium* species, three *Amoeba* species, three *Cryptosporidium* species, three *Giardia* species, five microsporidia species, three *Piroplasma* species, five *Toxoplasma* strains and four *Trypanosoma* species. By applying a reciprocal best BLAST hit approach, we determined the orthologous proteins among these genomes. For each orthologous group, we used MUSCLE v3.8.31 to do multiple sequence alignment (Edgar, 2004). Then we determined the lysine conservation for each species by counting the total number of conserved acetylated lysines and the total number of conserved non-acetylated lysines. A lysine residue was considered to be conserved if both the *P. falciparum* 3D7 protein and the query protein in the multiple sequence alignment are lysine residues at the aligned position. All lysine residues of the proteins identified in this study were considered as control. Mean conservation of the acetylated and control lysines between *P. falciparum* sequences and sequences from other microorganisms in the EupathDB were plotted separately. *P*-values were calculated for each comparison using Fisher's exact test. To determine the level of conservation of acetylation in the glycolysis pathway, sequences of 10 acetylated enzymes found in the survey were aligned using CLUSTAL W with their known acetylated orthologues in nine organisms (http://www.ebi.ac.uk/Tools/msa/clustalo/). Acetylation in *P. falciparum* was considered conserved if the acetyllysine was also found in one of the orthologues at the aligned position.

Statistical analysis

Statistical tests for potential over- and under-representation of protein functional groups were conducted using the hypergeometric distribution (Khatri and Draghici, 2005). The significances of lysine conservation were analysed by Fisher's exact test for each species separately using the R software.

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