A Global Survey of ATPase Activity in Plasmodium falciparum Asexual Blood Stages and Gametocytes*§

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Effective malaria control and elimination in hyperendemic areas of the world will require treatment of the Plasmodium falciparum (PF) blood stage that causes disease as well as the gametocyte stage that is required for transmission from humans to the mosquito vector. Most currently used therapies do not kill gametocytes, a highly specialized, non-replicating sexual parasite stage. Further confounding next generation drug development against PF is the unknown metabolic state of the gametocyte and the lack of known biochemical activity for most parasite gene products in general. Here, we take a systematic activity-based proteomics approach to survey the activity of the large and druggable ATPase family in replicating blood stage asexual parasites and transmissible, non-replicating sexual gametocytes. ATPase activity broadly changes during the transition from asexual schizonts to sexual gametocytes, indicating altered metabolism and regulatory roles of ATPases specific for each lifecycle stage. We further experimentally confirm existing annotation and predict ATPase function for 38 uncharacterized proteins. By mapping the activity of ATPases associated with gametocytogenesis, we assign biochemical activity to a large number of uncharacterized proteins and identify new candidate transmission blocking targets. Molecular & Cellular Proteomics 17: 10.1074/mcp.RA117.000088, 111–120, 2018.

Despite tremendous treatment and control efforts, malaria continues to be the world’s most devastating parasitic disease, causing more than 200 million clinical cases and 400,000 deaths in 2014 (1). The Anopheles mosquito vector sustains high levels of reinfection in endemic regions and severely impedes the success of curative drug therapy for malaria elimination (2). As a result, efforts to eliminate malaria must include strategies that prevent parasite transmission. Such efforts have historically focused on vector control and the use of mosquito bed nets (3) but less on preventing parasite transmission from humans to mosquitoes. Because only the asexual blood stages of the parasite cause disease, they are the main target of most antimalarials. In contrast, the developmental stages responsible for transmission are gametocytes; male and female sexual stages that differentiate from blood stage parasites with limited frequency, mate when taken up by a mosquito, and successfully infect the mosquito vector. Most drugs that are effective against the asexual blood stages are ineffective against gametocytes, suggesting that a better understanding of the distinct biology of gametocytes is important for the identification of transmission-blocking drug targets (4). Recent transcriptional and proteomic studies have begun to define the molecular underpinnings of gametocyte development (5, 6). However, transcript and protein abundance are only approximations of biochemical activity, which eventually determines biological function and druggability of a target.

An additional hurdle for drug development is the lack of reliable experimental annotation for most Plasmodium gene products. Although sequence-based annotation predicts functions for large numbers of gene products, species-specific proteins often remain uncharacterized. As a result, large parts of most genomes lack even the most basic experimental annotation (7), especially in phylogenetically deep-branching organisms such as Plasmodium parasites that have no closely related classical model organisms. Knowledge of a protein’s biochemical activity can accelerate target-based drug discovery, and identifying the biochemical activity of species-specific proteins is a particularly useful contribution to accessing an organism’s druggable enzyme space. Species-specific proteins often underlie an organism’s pathogenesis and their
lack of homologs allows for therapeutic targeting with minimal toxicity. Activity-based approaches are required for such sequence-independent, experimental identification of biochemical activity and annotation (8). Activity-based protein profiling (ABPP) has found application in the mapping of metabolic activity and the annotation of proteomes in several organisms (9, 10). ABPP directly reports on specific catalytic activity of an enzyme family in the context of the entire native proteome. In this way, ABPP surveys proteomes on the level most relevant for biological function and drug development.

ATP hydrolysis by ATPases supports virtually every cellular function, and ATP hydrolyzing activity generally makes an enzyme susceptible to inhibition by ATP analogs, an important class of pharmaceutical compounds. Here, we used ABPP with an ATP-based activity probe in combination with quantitative mass spectrometry to survey the ATPase activity in the proteome of Plasmodium falciparum (Pf), the most important human malaria pathogen. We surveyed both asexual and sexual stage parasites to identify stage-specific ATPase activity and activity that can be targeted in both the disease-causing and transmissible stages. In addition, we confirm experimental annotation of 141 Pf ATPases and predict ATPase activity for 37 uncharacterized proteins. Despite the reduced metabolic activity of non-proliferative sexual stage gametocytes, we show that many ATPases remain active in gametocytes and provide potential transmission blocking targets.

**EXPERIMENTAL PROCEDURES**

**Parasite Culture and Gametocyte Differentiation—**P. falciparum strain NF54 blood stage cultures were produced in complete medium consisting of RPMI 1640 (25 mM HEPES, 2 mM L-glutamine, 50 μM hypoxanthine, and 10% A+ human serum) and 5% O+ erythrocytes in an atmosphere of 5% CO2, 5% O2 and 90% N2. Late-stage infected erythrocytes were enriched using a Percoll gradient (60% Percoll in 1× PBS). Following three washes with 1× PBS the pellets were lysed with 0.2% saponin, washed three times with 1X PBS and stored at −80 °C. Gametocyte cultures were initiated at 6% hematocrit and 0.75–1% parasitemia (mixed stages) and maintained with daily complete medium changes. Asexual blood stages were depleted by supplementing complete media with heparin (20 U/ml) on days seven through nine. On day 11 the gametocytes were enriched by MACS LD column. The column eluant was pelleted and washed three times with 1× PBS. Pellets were lysed with 0.2% saponin, washed three times with 1× PBS and stored at −80 °C.

**Preparation of Pf Cell Lysate—**Frozen cell pellets were resuspended in 1× PBS with protease inhibitor mixture (SIGMAFAST) and then freeze-thawed 3 times to facilitate lysis. Then, samples were sheered with a 20-gauge needle. Insoluble material was pelleted by centrifugation and the supernatants were collected. Protein content was measured by Bradford assay and quality of lysate was determined by SDS-PAGE followed by Coomassie staining.

**Probe Labeling, SDS-PAGE Analysis of Pf Cell Lysate, and ATPase Assay—**Lysates from schizont culture (50 μg total protein per sample) were treated with ATP-ABP (20 μM) or DMSO (no-probe control), vortexed and incubated for 1 h at 37 °C. Following probe incubation, lysates were treated with an azide-derivatized Cy7.5 fluorescent reporter group (30 μM), tris (2-carboxyethyl) phosphine (TCEP, 250 μM), tris[1-benzyl-1H-1,2,3-triazol-4-yl]methylamine (TBTA, prepared in 4:1 tert-butanol:DMSO, 250 μM), and CuSO4 (500 μM). The samples were vortexed and incubated at room temperature in the dark for 1.5 h. SDS-PAGE reducing loading buffer was added to the samples, heated at 95 °C for 5 min, and loaded onto a 4–15% Tris-Glycine gel. Gels were imaged using a Li-COR Odyssey imaging system. For measuring ATP hydrolysis, purified protein was diluted to 10 μM in PBS and incubated with 2 μM ATP at 37 °C. At each time point, 20 μl of sample was transferred into a LUMITRAC 200 96-well plate, mixed with 20 μl of Kinase-Glo Max reagent (Promega). After 5 min incubation at RT, the luminescence was read by a Centro XS LB960 luminometer with a 50 ms exposure time.

**Probe Labeling and Sample Preparation for LC-MS Analysis—**Pf lysates (700 μg total protein per sample) were treated with ATP-ABP (20 μM) or DMSO (no-probe control) for 1 h at 37 °C. Proteomes were then treated with biotin-azide (25 μM), TCEP (250 μM), TBTA (250 μM), and CuSO4 (500 μM). The samples were vortexed and incubated at room temperature in the dark for 1.5 h. Probe-labeled proteins were enriched on streptavidin resin, reduced with TCEP, and alkylated with iodoacetamide. Proteins were digested on-resin with trypsin and the resulting peptides collected for LC-MS analysis. For full details see the supplemental Experimental Procedures.

**LC-MS Analysis of Probe-labeled Samples by the AMT Tag Approach—**Proteomics data for ATP-ABP-labeled and DMSO no-probe control samples were generated and analyzed using the accurate mass and time (AMT) tag proteomics approach as previously described (11). For full details see the Supplemental Experimental Procedures. To identify a protein as specifically labeled by the ATP-ABP, we set the following criteria: (1) the protein exhibits a significant difference between the probe labeled and no-probe control as judged by ANOVA (p < 0.05) and (2) the peptide exhibits >4-fold higher abundance in the probe-labeled sample relative to the no-probe negative control.

**Experimental Design and Statistical Rationale—**Gametocyte and schizont parasite cultures were synchronized and quality-controlled by blood smear analysis of parasite morphology. All sexual and asexual blood stage cultures were >95% pure. For ABPP, we used a negative control, probe solvent-only (DMSO) sample to control for background click-chemistry binding. We measured six experimental replicates for each probe and no-probe sample. For the gametocyte negative control, we rejected one replicate because of clear technical problems. LC-MS and data analysis are detailed in supplemental Experimental Procedures. Positive protein hits were defined as follows: (1) Detection of at least one unique peptide. (2) At least 4-fold higher signal of the probe-labeled over the DMSO control sample. (3) A p value for the difference between probe and DMSO-labeled sample of <0.05 as measured by ANOVA. A total of 425 proteins met these criteria and were included in the further analysis (supplemental Table S1). To determine significant differences between peptide abundance between schizont and gametocyte samples, we used two statistical tests as previously described (12). A two-sample t test and a g-test were carried out on each peptide and protein to test for significance of quantitative and qualitative differences, respectively. A Benjamini-Hochberg multiple test correction was applied to account for the multiple hypothesis tests. There were 169 proteins significant at a q-value of less than 0.05, of which only proteins with at least a 2-fold change by t test were considered significant. The experimental design and statistical analysis is further detailed in the flow chart in supplemental Fig. S2.
Search Parameters and Acceptance Criteria—Peptide identifications were generated using the MSGF+ search engine (release version v10072 (6/30/2014) and Uniprot’s Plasmodium falciparum (isolate NF54) protein sequence database collection (released May 8, 2015) containing 5928 total protein entries for which peptide to protein matches were searched. The specificity of all proteases used to generate peptides was partially or fully tryptic. We did not limit the number of missed and/or nonspecific cleavages, but limited the maximum peptide length to 50 residues. Modifications considered for peptide search included variable oxidation of methionine. Mass tolerances for precursor and fragment ions was $+/-$ 20 ppm precursor tolerance in the MSGF+ CID scoring model. Estimation of FDR was calculated by MSGF+ at an FDR $< 1\%$.

**RESULTS**

**ATPase Activity in Plasmodium falciparum**

**ATPase Activity Based Probe (ABP) Labels Many Pf Proteins—**To test the reactivity of the ATP activity based probe (ATP-ABP) (Fig. 1A, (13)) in the proteome of asexual erythrocytic schizonts and sexual gametocytes of Pf strain NF54, we labeled lysates of each with the ATP-ABP followed by conjugation with a fluorophore by click chemistry. We separated the samples by SDS-PAGE and imaged for fluorescently labeled proteins. The ATP-ABP reacted with many proteins across the molecular weight spectrum and showed large differences in the banding pattern between schizonts and gametocytes (Fig. 1B). ATP inhibited probe binding in a dose-dependent manner and led to complete inhibition at 2 mM. GTP and dATP, two closely related nucleotides, had little or no effect on ATP-ABP probe labeling at 2 mM, respectively, showing that the ATP-ABP probe distinguishes even among closely related nucleotide binding sites (Fig. 1C).

**ABPP-MS Predicts 425 Pf ATPases—**To systematically identify the ATPases in the Pf proteome, we next labeled Pf gametocyte and schizont lysate with ATP-ABP, and conjugated...
gated the probe with biotin. After enrichment using streptavidin, we digested labeled proteins on-bead using trypsin. The eluted peptides were then analyzed by LC/MS and quantitated by the AMT approach (11). Importantly, because of the activity-based labeling step prior to peptide abundance measurements, peptide abundance directly reflects ATPase activity. To control for background binding of click chemistry reagents in the schizont and gametocyte samples, we treated samples with the probe’s solvent DMSO only instead of probe prior to the click chemistry reaction. We applied three criteria to define positive hits: (1) Detection of at least one unique peptide. (2) At least 4-fold higher signal of the probe-labeled over the DMSO control sample. (3) A \( p \) value for the difference between probe and DMSO-labeled sample of \( <0.05 \) as measured by ANOVA. A total of 425 proteins met these criteria and were included in the further analysis (supplemental Table S1). We next plotted the Z-score of each measurement to compare reproducibility of replicates and the overall separation from probe versus no-probe samples. A heatmap representation of the ATP-ABP labeled proteins from the schizont sample showed robust signal and reproducibility across replicates (Fig. 2). The same representation of the gametocyte sample is provided as supplemental Fig. S1. A summary of the statistical analysis is shown in supplemental Fig. S2.

Many Hits are Known ATPases—We next compared our data with existing annotation from PlasmoDB. Because the annotation of Pf strain 3D7 is more current than that of NF54, we identified the 3D7 homologs of NF54 proteins and used the 3D7 annotation for all further analysis. We detected 388 previously annotated and 37 proteins of unknown function that met our significance criteria, a similar ratio of annotated proteins to proteins with unknown function as in the genome overall. The probe labeled a spectrum of proteins with different nucleotide binding characteristics. We detected 141 known ATPases such as Hsp70 and 90, T-complex protein 1, and several tRNA ligases. The ATP-ABP was initially designed to probe kinases. Indeed, we detected 16 kinases in our dataset, such as AGC kinase, casein kinase II subunit beta, nucleoside diphosphate kinase, pyruvate kinase, a probable ATP-dependent 6-phosphofructokinase, hexokinase, and phosphoglycerate kinase (supplemental Table S1, tab 2). Interestingly, we identified EXP2 and PTEX150, both of which complex with HSP101 to form the Plasmodium Translocon of Exported Proteins (PTEX) (14, 15). Because sample processing involves denaturation and breaking of protein-protein interactions by SDS, detection is only expected for directly labeled ATPases. Neither EXP2 nor PTEX150 has previously been associated with ATPase activity, but they may contribute to PTEX transport by ATP hydrolysis.

Consistent with previous studies, we also detected proteins that recognize the adenosine substructure in other chemical contexts, such as NAD-, FAD-, and acetyl-CoA-binding proteins, as well as DNA and RNA binding proteins, indicating some promiscuity of probe binding as reported previously (9, 16, 17). Some DNA and RNA binding proteins likely bind the probe in lieu of DNA- or RNA-associated adenosine nucleotides, whereas others, such as DNA and RNA helicases, also hydrolyze ATP. We further detected 11 acylphosphate-reactive proteins (Fig. 3A). In all, we detected 295 proteins, or \( \approx70\% \) of all hits, with existing annotation that is consistent with binding to ATP or closely related nucleotides. These data show that our screen reliably detects ATPase activity. There are several possibilities for detection of hits in our screen that are not predicted to bind ATP: Besides false positives, between 5–40\% of genes are estimated to be mis-annotated (18) and might indeed be ATPases. Also, multidomain proteins might have ATPase function but be annotated based on the function of a different domain. Our data should provide impe-
Several Uncharacterized Proteins Have ATPase Activity—Approximately 10% of Pf genes currently lack any annotation, and the annotation of many more Pf proteins is limited to the assignment of cellular localization, stage-specific expression, or presence of a known protein domain. Because ABPP recognizes biochemical activity independent of sequence and detects even the most divergent members of an enzyme family, our approach is particularly useful to identify biochemical activity among these uncharacterized proteins. Indeed, we detected 37 previously uncharacterized proteins in our screen (supplemental Table S1). To further test the idea that these proteins are ATPases, we next searched for GO terms that are associated with adenosine nucleotide binding. Four of the uncharacterized proteins were linked to GO terms that include ATP binding, and five more with binding to other nucleotides. Structural homology is generally a better indicator for functional similarity of two proteins than sequence homology, as structure is under stricter evolutionary constraints (19). To further test the group of uncharacterized proteins for indications of ATP binding, we generated structural predictions using the Phyre server (20). Of the uncharacterized proteins with no nucleotide binding-associated GO terms, three of the predicted structures additionally suggested nucleotide binding. The remaining hits without adenosine binding-related GO terms, sequence, or structural similarity likely comprise highly divergent ATP binders and false positives. Mining of these uncharacterized proteins further is likely to uncover additional unusual adenosine nucleotide binders. For the many other Pf genes with very limited annotation, our data provide an additional level of functional information (supplemental Table S1).

PF3D7_0626700 Has ATPase Activity—The identification of many proteins of unknown function with no discernible sequence similarity to known ATP binding proteins or domains suggests the presence of noncanonical ATPases in Pf. To test whether these unusual proteins indeed bind and hydrolyze ATP, we chose the soluble uncharacterized protein PF3D7_0626700 for further analysis. This protein belongs to the uncharacterized protein family UPF0160 with orthologs across lower and higher eukaryotes. We recombinantly expressed the protein in fusion with a SUMO tag in E. coli and purified the protein by metal affinity and size exclusion chro-

Fig. 3. Comparison of ATPase screen with existing annotation. A, The pie chart shows the functional classification of hits according to current annotation from PlasmoDB. B, Gel analysis following ABPP and addition of a fluorescent tag shows ATP-ABP binding to PF3D7_0626700 and concentration-dependent competition of probe binding by ATP. C, ATPase assay shows ATP hydrolysis over time by SUMO-tagged PF3D7_0626700, the positive control Ser/Thr kinase Rv0931c, but not the unrelated Mycobacterium tuberculosis SUMO-tagged protein Rv2141.
matography. In a gel assay, PF3D7_0626700 bound the probe, and ATP competed with probe binding in a concentration-dependent manner (Fig. 3B), confirming ATP binding. Of note, we observed only minimal contamination by *E. coli* proteins that also bound probe, showing that the prep was largely free of ATPase contaminants. To test for ATP hydrolyzing activity, we tested PF3D7_0626700 in a luciferase-based ATP assay. PF3D7_0626700 consumed ATP over time with slightly faster kinetics than a *Mycobacterium tuberculosis* Ser/Thr kinase control (Rv0931c, Fig. 3C). The unrelated *Mycobacterium tuberculosis* protein Rv2141 that we also expressed with an N-terminal SUMO tag did not show any ATP degradation. Together, these data indicate that PF3D7_0626700 is a novel, noncanonical ATPase. Orthologs of PF3D7_0626700 occur in many eukaryotic species including in humans. The human ortholog Melanocyte proliferating gene 1 (Mig1) has 44% sequence identity over 84% of the sequence. These data raise the possibility that other or even all members of the family are in fact ATPases.

**ATPase Activity Changes from Asexual to Sexual Development**—Uncommitted asexual schizonts sustain blood-stage infection whereas differentiated sexual stage gametocytes are required for transmission to the mosquito vector. To compare ATPase activity in these stages, we used an experimental setup as shown schematically in Fig. 4A. We detected large changes in peptide abundance measurements between schizonts and gametocytes (Fig. 4B). In some instances, no peptides could be detected in one of the two stages. These peptide measurements are either missing because of random experimental error or because of a biological effect. To limit the impact of randomly missing data, and to distinguish between experimental error and biological effect, we measured six experimental replicates for each sample. We then applied two statistical tests that give two measures of significance based on peptide intensities (t test) and peptide occurrence (g-test). We used a standard ANOVA to estimate variance and significance of differences between schizonts and gametocytes when data in both stages were available for all replicates. For proteins with missing data, we used a modified χ² test, or g-test, which examines the pattern of missing data in one sample compared with that in all samples to determine significance without a quantitative determination of the fold change. We considered peptides significant if they passed either test; however, we only calculated fold changes for proteins with complete data. Although fold change was calculated only for this group of proteins, the group with missing data that showed significant changes by g-test likely comprises many of the enzymes with the largest activity changes, as no peptide was measured in as many as all replicates, indicating complete loss of activity. We included these proteins in tab 2 of *supplemental Table S2* and sorted these hits by q-value. We detected 169 proteins with significant differences between schizont and gametocyte by at least one of the tests. Of these, 89 activities were increased in the schizont and 80 were decreased compared with the gametocyte. The significant proteins for which a fold-change could be calculated are further shown as a volcano plot in Fig. 4C. All proteins with significant changes by both tests are listed in *supplemental Table S2*.

In total, nearly 40% of the ATP-ABP labeled proteins showed activity changes between asexual schizonts and sexual gametocytes, reflecting the unique biology of each life cycle stage. The stage-specific gamete antigen 27/25 (Pfg27) (21) was overrepresented 500-fold in the gametocyte, providing an internal control for successful gametocyte differentiation. The role of Pfg27 in gametocyte development is somewhat unclear, with one study showing that Pfg27 is required (22), and another that it is dispensable for gametocyte development (23). Except for RNA binding activity, the biochemical activity of Pfg27 is unknown (24). We have previously shown that the ATP-ABP can label some RNA-binding proteins in lieu of RNA (25). Alternatively, Pfg27 may have ATPase or GTPase activity, as also suggested by GO terms associated with the protein. In addition, 23 proteins with unknown function showed more than 2-fold change, 10 with higher abundance in schizonts, and 13 in gametocytes. These proteins may play important roles in gametocyte development and are candidates for further testing of their role during the respective life cycle stage.

Differences in ATPase activity of proteins between the two stages highlight the different cellular processes necessary for each lifecycle stage. For example, the schizont showed overrepresentation of twelve 40S and 60S ribosomal proteins. Protein translation consumes a large share of cellular ATP and increased ATPase activity of ribosomal proteins likely reflects higher rates of protein translation in schizonts compared with the non-proliferating gametocytes. This lack of proliferation poses challenges and limits the druggable space in gametocytes, as drugs often target active growth and division. The DOZI helicase is essential for the storage of mRNA in translational repressor complexes in female gametocytes (26). These mRNAs are stored for translation after gamete formation and are required for fertilization in the mosquito. Our data suggest that this essential process is already ongoing in stage III gametocytes, or that DOZI has additional functions in earlier gametocytes, as has been previously suggested (26). To test whether our analysis predicts metabolic differences between the two stages, we analyzed all up- or downregulated probe-labeled proteins by mapping them to metabolic pathways using BioCyc (27). Two nucleotide binding proteins overrepresented in gametocytes map to the citric acid cycle, succinyl CoA ligase and malate dehydrogenase. The overrepresentation of these enzymes is 10 and 15-fold, and suggests that gametocyte development is accompanied by higher flux through the citric acid cycle. The ATP-dependent RNA helicase DDX6/DOZI was also overrepresented in the gametocyte.
Fig. 4. ATPase activity changes associated with the schizont-gametocyte transition. A, Overview of comparative ABPP-MS strategy. B, The ATPase activity in Pf lysate from schizonts and gametocytes was measured by ABPP-MS. Heatmap representation shows Z-scores of peptide abundances. C, Volcano plot representation of the activity changes between schizont and gametocyte.
Although specific targeting of gametocytes by antimalarials using combination therapies is a plausible approach, targeting proteins that allow for cross-stage inhibition are also desirable. Our analysis shows several ATPases that are present and active in both asexual schizont and sexual gametocytes. These include several kinases such as cAMP-dependent protein kinase and adenylate kinase, suggesting a cross-stage housekeeping function. Similarly, several HSPs, including HSP60, 70, 90, and 110 maintained ATPase activity in schizont and gametocyte, suggesting essential functions in both lifecycle stages that could be chemically vulnerable.

**DISCUSSION**

Large parts of the *Pf* genome remain uncharacterized and this lack of annotation remains a fundamental hurdle for malaria research. There are many levels of annotation from assigning basic biochemical activity to a complete description of a gene product’s function in the cellular context. Although sequence homology-based annotation is usually the first step in annotating genomes, it only predicts and inherently does not uncover truly new protein function. Functional drift through small amino acid changes and the large number of species-specific proteins further confound sequence-based annotation. Consequently, experimentation is essential to validate sequence-based annotation and to discover new protein function; however, high-throughput experimental annotation to discover biochemical activity of proteins remains exceedingly difficult. This limitation particularly impedes drug development, as knowledge of biochemical activity is often a prerequisite for target-based drug development. The annotation of *Pf* and related apicomplexan parasites has been particularly challenging in this respect, as their distinct and somewhat isolated phylogeny relative to model organisms has limited homology-based annotation transfer, and the difficulties of genetic manipulation in this species has further slowed experimental annotation, resulting in 10% of gene products with no and 40–50% with minimal annotation (28).

ABPP is emerging as a powerful approach for parallel experimental annotation. ABP probes are designed so that their labeling of a target protein is evidence of a particular catalytic activity of that protein. ABPP-based annotation, however, is only as good as the probe and its selectivity. The ATP-ABP used in this study is particularly suited for annotation purposes because the probe is based on the natural ligand ATP. The acylphosphate-based ATP-ABP was originally developed for the detection of kinases, but it labels ATPases more broadly, making it a useful tool to survey this large group of enzymes in general. Although an annotation derived from ABPP reports directly on biochemical activity, the characterization of an enzyme as ATPase is still broad and includes a diverse group of enzymes. Off-target binding of the ATP-ABP can lead to the detection of proteins that recognize other adenosines closely related to ATP. Further, some proteins that bind but do not hydrolyze ATP are likely also labeled if a sufficiently nucleophilic residue is in the vicinity of the ATP binding site. This might be the case for RNA and DNA binding proteins that potentially present many probe binding sites across the protein. Although these probe properties limit the use of the probe, the strength of our predictions of ATPase activity are still likely high. Based on the number of labeled proteins with known function that are consistent with ATPase activity (~70%), we estimate that most labeled uncharacterized proteins are also true positives. We identified 37 uncharacterized proteins as ATPases and confirmed ATP hydrolyzing activity for one of them. Several of those uncharacterized proteins are associated with GO terms related to ATP binding, further suggesting high fidelity of our predictions. Surprisingly, we detected none of the ~100 predicted Ser/Thr protein kinases (29), although at least 14 of those have previously been detected by proteomics (30). Many of these kinases, which are thought of as potential drug targets, may in fact be largely inactive in bloodstage parasites.

Several proteins previously annotated as ATPases were not identified in our screen. One of these proteins is HSP101, which together with EXP2 and PTEX150 forms the *Plasmodium* translocon of exported proteins (PTEX), which is important for export of proteins into the host erythrocyte (31, 32). EXP2 forms a transmembrane pore within the parasitophorous vacuole membrane, which allows export of substrates into the erythrocyte cytosol. PTEX150 links EXP2 with HSP101. HSP101 has been thought to be the protein important for providing energy to the translocation of proteins, yet Mesen-Ramirez et al. recently showed that in fact, substrate proteins appear to be in direct contact with EXP2, not HSP101, suggesting that EXP2 may have translocon activity itself (32). This report is consistent with our data that show that EXP2 and PTEX150 have ATPase activity, whereas HSP101 may not. Although HSP101 is necessary for protein export, EXP2 and PTEX150, not HSP101, may provide the energy for translocation.

Transmission from the human to the mosquito host requires *Pf* development into gametocytes. The cell cycle-arrested male and female gametocytes are poised for uptake during a mosquito blood meal, after which the parasite continues its life cycle in the mosquito. The transmission-competent gametocytes are of major interest, as interfering with transmission is increasingly becoming a priority in the effort to eradicate malaria (33). The differences between asexual and sexual stage parasites are complex and reflected on a molecular level by many stage-specific proteins in the proteome (34). Gametocyte development progresses through five morphologically different stages. We chose stage three gametocytes for our studies to identify factors that drive differentiation rather than markers of terminal differentiation. In fact, several of the proteins identified as ATPases are important for lifecycle transitions in the mosquito, suggesting these proteins are vulnerable targets both in the human blood as well as the mosquito midgut. These proteins include Pfg27, Pfs48/45, and the
plasma membrane associated protein Pf230 (35). Pf648/45 assembles on the periphery of the parasite following transmission and is important for adhesion. Interestingly, Pf648/45, a 6-Cys protein, is a transmission blocking vaccine candidate (36). The activity changes we observed affected many pathways, for example the TCA cycle, indicating a possibly essential shift in metabolism toward the TCA cycle in gametocytes. Indeed, genetic studies have shown that although the TCA cycle is dispensable in asexual blood stages, it is essential in gametocytes (37) and for parasite development in the mosquito.

Our study emphasizes that schizonts and gametocytes are very distinct parasites and require separate consideration for drug development. Patients can be clinically asymptomatic but still carry gametocytes and transmit the parasite. Similarly, antimalarial drugs strongly bifurcate into schizontidal and gametocytocidal, with only few drugs such as artemisinin derivatives and 8-aminoquinolines killing both stages (4). This distinct activity profile and transmission without clinical disease reflect the large differences between these stages and the underlying cellular programs and underscores the need for identifying targets specifically in gametocytes. However, there have been few systematic studies aimed at identifying gametocyte targets. Several studies have identified transcriptomic or proteomic correlates of different life cycle stages (5, 6). However, most ‘omics approaches do not report on biological activity of gene products and are only an approximation of true activity. A unique advantage of ABPP-MS is that it allows direct measurement of biochemical activity, which is arguably the most relevant parameter for understanding biology and drug development. Furthermore, we identify the ATPases that remain active or are specifically regulated in gametocytes and that could generally be amenable to inhibition by ATP analogs. Thus, this study provides a high-content, systematic survey of enzyme activity in Pf that contributes extensive new experimental annotation, direct measurement of activity changes associated with transmission-relevant life cycle transition, and, by profiling ATPases, provides targets for drug development through ATP analogs.

Acknowledgments—We thank the employees of the Center for Infectious Disease Research for generously supporting large parts of this work through donations to the 2013 employee giving campaign.

DATA AVAILABILITY

The mass spectrometry data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the following: Project name: ATPase activity in Plasmodium falciparum asexual blood stages and gametocytes. Project accession (PRIDE): PXD005335. Project DOI: 10.6019/PXD005335.

ATW, LNA, NCS, and RDS were supported by the NIH Biomedical Technology Research Resource for Integrative Biology (NIGMS P41 GM103493). Significant portions of this work were performed in the Environmental Molecular Science Laboratory, a DOE/BER national scientific user facility at Pacific Northwest National Laboratory (PNNL). PNNL is operated for the DOE by Battelle under contract no. DE-AC05-76RL01830. AF was supported by NIH grant AI055396. This article contains supplemental material.

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